THE MAINTENANCE OF VARIATION IN CYPRINODONTIFORMES

BY

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DISSERTATION

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ABSTRACT

Maintenance of genetic variation in the face of strong natural selection is a long-standing problem in evolutionary biology. Two extreme examples of this are the guppy (*Poecilia reticulata*) and the bluefin killifish (*Lucania goodei*), two freshwater species in the order Cyprinodontiformes with extensive within-population color pattern variation found among males. I use these two species as case studies to examine the evolution and maintenance of color pattern variation.

First, I examine a potential reason behind the evolution of a female mating preference for males with novel color patterns in guppies. This preference has been implicated as a factor in maintaining the genetically-determined color pattern polymorphisms found in male guppies, and inbreeding avoidance has been proposed as a mechanism to explain its evolution. Inbreeding avoidance is advantageous when populations exhibit inbreeding depression and the opportunity for mating between relatives exists. To determine whether these conditions are met in a natural guppy population, I assessed mating and reproductive patterns using parentage analysis. Females produced more offspring with less-related males than with more-related ones. In addition, females were more likely to have mated with less-related males, but this trend was only marginally significant. Male heterozygosity was positively correlated with mating success and with the number of offspring sired, consistent with strong inbreeding depression for adult male fitness. These results show that strong inbreeding depression occurs in guppies, and individuals tend to avoid mating with relatives. Thus, the preference for novel male phenotypes may have evolved due to the advantage inherent to avoiding inbreeding.

In my remaining chapters, I focus the bluefin killifish (*Lucania goodei*). These fish exhibit extensive color variation in their fins, but the function of this variation has not yet

been determined. I collected males from multiple populations across Florida and used absorption spectroscopy to identify the pigments responsible for the fin coloration. I determined that orange coloration in the caudal fin was caused by a carotenoid pigment. Color in the anal fin was either pterin based (yellow and red) or structural (blue) with a melanic fin border. Using a behavioral assay designed to measure dominance, I sought to determine the informational content of each pigment. Black melanic markings on the anal fin were strongly related to dominance. Aggression was greater between males of similar sized melanic stripes, indicating that they functioned as badges of status in territorial interactions with other males. In keeping with their dietary origin, caudal carotenoid levels positively correlated with condition but did not influence dominance interactions. However, the highly labile ornament predicted parasite infection and spawning success, suggesting a role in intersexual selection, with caudal carotenoid as a signal of health to potential mates. Similarly, pterin pigmentation in the anal fin, while not related to dominance, was related to overall spawning levels and parasite infection, suggesting that pterin pigmentation may also signal immune status.

In order to test whether the variation found in fin color has effects on fitness, I set up a breeding experiment in which I examined the role of the anal fin polymorphism and the amount of pterin, melanin, and carotenoid pigmentation in each male. I manipulated anal fin morph ratios in breeding populations housed in a greenhouse to determine if morph rarity conferred a fitness advantage, as determined by identifying fry paternity. I found no evidence of negative frequency-dependent selection on anal fin morph. However, red morph males did sire more offspring on bottom spawning substrates when rare. This suggests lighting environment may affect female preference and influence morph abundance. In addition to noting morph effects, I also tested the effects of level of pigmentation on male fitness. I

demonstrated that males with more anal fin pigmentation (both pterin and melanin) and caudal fin pigmentation (carotenoid) sired more offspring.

Finally, I examined the interplay of lighting environment, visual system oscillations, and color preferences in *Lucania goodei*. I measured the diurnal pattern of cone opsin gene expression in the bluefin killifish to see if overall or proportional opsin expression was tuned to match the daily blue-shift in light at dawn and dusk. LWS, RH2-1, RH2-2, and SWS2B (but not SWS1 or SWS2A) opsin gene expression was lowest at midnight and dawn and highest at midday and dusk, and the observed temporal shifts were many times larger than an accompanying difference in production of opsins in tannin-stained versus clear water habitats. I also measured color preference in a foraging assay at dawn, midday, and dusk to determine if opsin gene expression influenced fish behavior. Rather than correlating with opsin expression, foraging behavior matched lighting conditions, with higher preferences for blue at noon and red at dawn/dusk, when these wavelengths are comparatively scarce and the contrast of these colors is increased. My results suggest that *L. goodei* exhibit strong diurnal cycles of opsin expression but that these are not correlated with light intensity or light color per say. Temporally variable preferences for different colors are probably the result of lighting environment rather than opsin production.

The results from the studies on *Lucania goodei* suggest that the color pattern variation observed in this species is affected by several factors. The continuous variation found in degree of pigmentation (anal fin melanin, anal fin pterin, and caudal fin carotenoid) reflect variation in condition or fighting ability. In contrast, lighting environment may strongly influence the red/yellow/blue polymorphism found in the anal fin. When red wavelengths are rare, as at dawn and dusk and in deep waters, red males may have a mating advantage over blue and yellow males. When blue wavelengths are rare, as in tannin-stained waters and at

noon, blue males may have a mating advantage. Thus, temporal and microhabitat variation may contribute to the maintenance of the anal fin polymorphism.

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CHAPTER 1

INTRODUCTION

The critical role natural variation plays in evolutionary processes has been recognized since Charles Darwin's time. In fact, Darwin's *On the Origin of Species* (1859) defines natural selection as the "preservation of favourable variations and the rejection of injurious variations." Variation powers the process of adaptation, without which, species are doomed to rapid extinction. Biologists frequently evoke the image of Alice and the Red Queen in Lewis Carroll's *Through the Looking Glass* to illustrate this principle of never-ending evolution. Alice wonders why her constant running is getting her nowhere and is told by the Red Queen, "Now, here, you see, it takes all the running you can do, to keep in the same place." But with this concept of perpetual directional evolution comes a problem: if organisms are rapidly running through their supply of variation in the evolutionary arms race, how is it possible that they are able to remain so variable? Nature presents us with a sustained bounty of individual variation in genetics, morphology, physiology, and behavior across a wide range of taxa. Understanding the processes that maintain this diversity against the strong push of selection is one of the main goals of evolutionary biology.

While mutation is ultimately the source of all variation, it seems unlikely to be the sustaining supply of variation in traits that have neither a high mutation rate nor a large number of controlling genes (reviewed in Radwan 2008). Thus, scientists have sought to identify the elements that maintain variation. Much research, including my own, has focused on the most conspicuous manifestation of natural variation: the diversity of animal coloration. Color patterns in animals are subject to selection for camouflage, aposematism, mimicry, thermoregulation, and mate attraction; however, color-variant individuals still persist (Protas

and Patel 2008). This variation can come in distinct classes (polymorphisms) or fall along a continuous gradient. Variation *between* populations can be explained by local adaptation, but *within* a population, where one might expect a single optimum to exist, we still find extensive diversity.

There are multiple ways in which variation in coloration might be maintained. Negative frequency-dependent selection is the best supported of these mechanisms and occurs when fitness is inversely correlated with morph abundance. In this case, males with rare coloration may have an advantage, such as in guppies, where females prefer rare or novel morphs in mate choice experiments (Hughes et al. 1999; Zajitschek et al. 2006). Alternately, it may be that common coloration comes with costs, as in the clonal interference of the cichlid fish *Neochromis omnicaeruleus*, where there is increased aggression from similar morphs (Dijkstra et al. 2008). Predators may also form search images that favor common morphs and selectively prey upon them (Olendorf et al. 2006). For negative frequency-dependent selection to operate, selection does not have to act directly on appearance. For example, in side-blotched lizards, there are three mating strategies, each of which is superior to one other strategy. The behavioral types are linked to color patterns, and as a result, color variation is maintained via rock-paper-scissors selection on mating strategy (Sinervo and Lively 1996).

Other proposed mechanisms for the maintenance of variation in color patterns are less well supported but deserve consideration (reviewed in Roulin and Bize 2007; Radwan 2008). For example, differences between microhabitats that result in fitness differences between morphs may be able to maintain diversity (Chunco et al. 2007; Skualson and Smith 1995), especially when morphs exhibit habitat choice (Pamilo 1988). Under even more restrictive conditions, temporally fluctuating selection may also maintain genetic variance (Hedrick 1986; Elner and Hairston 1994). Spatial and temporal variations rely on fluctuations in the

direction of selection, but when selection is always in the same direction, it becomes difficult to maintain variation. This is especially common in sexually selected characters. Theorists believe color patterns like those found in secondary sexual characters still exhibit variation because their expression is tied to the quality of the individual (reviewed in Searcy and Nowicki 2005). Females use these traits to assess males and have a preference for greater values of the traits, but not all males exhibit high values because they are not in superior enough condition to do so. Thus, as long as males vary in condition (e.g. they vary in access to food or parasite load), the ornaments will also vary.

Ultimately, of course, processes that maintain variation will be species- and trait-specific. For my dissertation work, I examine the forces maintaining diversity in Cyprinodintiformes, a diverse order of teleost fishes. I use the guppy (*Poecilia reticulata*), and the bluefin killifish (*Lucania goodei*) in my research. The males of both of these species are colorful, and the variation observed is maintained within populations. This makes them ideal systems in which to study the evolution of color pattern variation. In fact, the guppy has already been established as a model organism in evolutionary biology, and the bluefin killifish is rapidly becoming one.

Guppies have long been remarked upon for their extreme diversity and concurrent lack of speciation. One cause for this diversity is a female preference for rare and unfamiliar phenotypes (Farr 1977; Hughes et al. 1999; Zajitschek and Brooks 2008). It has been hypothesized that this preference for novelty may be derived from an avoidance of potentially deleterious inbreeding in isolated streams. However, no studies actually examined whether inbreeding avoidance was present in natural (rather than lab-raised) populations of guppies. In my first chapter, I examine a natural population for evidence of inbreeding avoidance. All the adults were captured from a single stream section in Trinidad, and the females were allowed to give birth. Because they are live-bearers, the resulting offspring were the result of

natural mating behaviors in the wild. Using microsatellite analysis, I was able to match offspring to their sires. I demonstrate that females had more offspring with less related males and preferred more heterozygous males. This suggests that mate preferences that result in inbreeding avoidance, such as those for rare males and rare male phenotypes, are highly advantageous.

My remaining chapters focus on the bluefin killifish. The bluefin killifish (*Lucania goodei*) is a small, common, freshwater fish that inhabits the swamps and streams of the southeastern United States (Page and Burr 1991). The mating system is promiscuous with no evidence of male parental care (Fuller and Travis 2001). Males are territorial, and females deposit a few eggs at a time across multiple males' territories. The males of the species are an eye-catching illustration of nature's variation. While all females sport the same drab coloration, the adult males have blue, red, or yellow coloration on their anal fins. The fish also vary in the amount of black pigmentation they harbor. More specifically, the amount of black outlining the distal end of the anal fin can range from a strong thick band to completely absent. The male caudal fin is also highly variable, having an orange coloration that can vary from almost nonexistent to deeply saturated. Thus, the color variation found in *Lucania goodei* varies continuously in the case of the caudal fin and the anal fin outline and categorically in the case of anal fin color.

At least two anal fin morphs are found per population (often collected in the same sweep of the dipnet) (Fuller 2002), and the diversity of this fin has been explored in previous research. The red/yellow anal fin polymorphism is inherited in a mendelian manner, with yellow (YY, Yy) being dominant to red (yy). The blue morph is orthogonal to the red/yellow dichotomy and is strongly affected by environment (Fuller and Travis 2004). The persistence of these two red/yellow alleles of large effect begs the question of what maintains this variation. In my third chapter, I work on identifying the pigments responsible for the fin

colorations under the premise that their developmental origins may supply information about their function, and by extension, their maintenance. I show that the killifish use carotenoid, pterin, melanin, and structural coloration in their fins. I show that pigmentation is correlated with behaviors and parasite levels and influences dominance and mating success. However, I do not detect an advantage for one anal fin morph over another. This experiment is the first to simultaneously measure the informational properties of melanic, pterin, and carotenoid ornaments in any organism.

In my fourth chapter, I use a mesocosm experiment to correlate pigmentation with number of offspring sired. By manipulating the ratios of red morphs to yellow morphs, I test if rare morphs have an advantage. I also test the influence of pigmentation on male paternity. I find no evidence for negative frequency-dependent selection, but red males seem to have a slight advantage in tanks where they are rare. Males with more pterin, melanin, and carotenoid pigmentation overall sire more offspring, reinforcing the notion that pigmentation plays an important role in fitness.

In my final chapter, I test the idea that temporal variation might play a role in maintaining variation. The quantity and quality of light varies over time, with light becoming blue-shifted at dusk and dawn. The fish visual cells responsible for color vision (cones) experience a natural diurnal rhythm of building during the day and shedding during the night. We sought to determine if this cone construction, as measured by expression of the main visual pigment (opsin) is matched to the daily fluctuations in light. We compare diurnal rhythms in light availability and opsin expression to behavioral color preference in a pecking assay. We show that relative opsin expression is unrelated to diurnal light patterns but is related to habitat-derived lighting differences. We show that the fish have a preference for red coloration at dusk and dawn, when those wavelengths are relatively rare. This suggests

that temporal variation may also help maintain diversity, especially if temporally variable color preferences translate into temporally variable preferences for male color.

Both guppies and bluefin killifish are similar in that they maintain a large degree of variation in color patterns. However, they are quite different in the methods by which that variation is maintained. In guppies, a female preference for novelty in parallel with increased predation on common morphs translates into extensive negative frequency-dependent selection. The story of bluefin killifish is less complete. Variation in pigmentation is related to variation in condition or fighting ability. Females do not exhibit a preference for rare males, but rather have a small preference for red males that varies somewhat with light. The way in which red, yellow, and blue morphs are able to coexist is still actively being explored.

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CHAPTER 2

INBREEDING DEPRESSION AND INBREEDING AVOIDANCE IN A NATURAL POPULATION OF GUPPIES (*POECILIA RETICULATA*)[1]

ABSTRACT

Maintenance of genetic variation in the face of strong natural selection is a long-standing problem in evolutionary biology. One of the most extreme examples of within-population variation is the polymorphic, genetically determined color pattern of male Trinidad guppies (*Poecilia reticulata*). Female mating preference for rare or novel patterns has been implicated as a factor in maintaining this variation. The origin of this preference is not understood, although inbreeding avoidance has been proposed as a mechanism. Inbreeding avoidance is advantageous when populations exhibit inbreeding depression and the opportunity for mating between relatives exists. To determine whether these conditions are met in a natural guppy population, we assessed mating and reproductive patterns using polymorphic molecular markers. Females produced more offspring with less-related males than with more-related ones. In addition, females were more likely to have mated with less-related males, but this trend was only marginally significant. Male heterozygosity was positively correlated with mating success and with the number of offspring sired, consistent with strong inbreeding depression for adult male fitness. These results provide

[1] This chapter appeared in its entirety in *Ethology* as Johnson, A.M., Chappell, G., Price, A.C., Rodd, F.H., Olendorf, R., & Hughes, K.H. 2010 Inbreeding Depression and Inbreeding Avoidance in a Natural Population of Guppies (*Poecilia reticulata*) 116:448-457. This article is reprinted with permission of the publisher and is available from http://onlinelibrary.wiley.com/ using DOI: 10.1111/j.1439-0310.2010.01763.x

substantial insight into mating patterns of a wild guppy population: strong inbreeding depression occurs, and individuals tend to avoid mating with relatives.

INTRODUCTION

The 'paradox of variation' remains an unsolved problem in evolutionary biology (Lewontin 1974). Natural selection tends to erode genetic variation (Fisher 1930; Lewontin 1974), yet high levels of polymorphism persist within species and even within single populations (Lewontin 1974; Houle 1992). This situation is especially paradoxical for heritable variation affecting traits closely tied to reproductive success or survival because these traits experience strong selection (Charlesworth 1984). Possible solutions to the paradox include continual input of new mutational variation and the active maintenance of variation by forms of 'balancing' natural selection.

Inbreeding avoidance can play a role in the maintenance of genetic variation by increasing heterozygosity in offspring. Inbreeding avoidance mechanisms are common in both plants and animals (Jarne & Charlesworth 1993; Pusey & Wolf 1996). In animals, inbreeding avoidance can be achieved by active behavioral avoidance of mating with relatives (e.g. Frommen & Bakker 2006; Lihoreau et al. 2007), by dispersal mechanisms (e.g., Berg et al. 2009), and by post-copulatory mechanisms of sperm precedence or biased sperm use (e.g. Tregenza & Wedell 2002; Bilde et al. 2007; Bretman et al. 2009).

Behavioral avoidance of inbreeding has been speculated to be a cause of a well-supported mating preference in guppies (*Poecilia reticulata*): female preference for males with unfamiliar or rare color patterns (Farr 1977; Hughes et al. 1999; Zajitschek et al. 2006; Zajitschek & Brooks

2008). Guppies exhibit inbreeding depression in laboratory experiments, including effects on offspring number and male development rate (Pitcher et al. 2008), juvenile survival and salinity tolerance (Nakadate et al. 2003), male courtship behavior (van Oosterhout et al. 2003; Mariette et al. 2006), male mating success (Mariette et al. 2006; Zajitschek et al. 2009), male ornamentation (Sheridan & Pomiankowski 1997; van Oosterhout et al. 2003; but see Mariette et al. 2006), and female size and fertility rate (van Oosterhout et al. 2007). In addition, guppies in many locales inhabit streams that have a pool-and-riffle structure (Reznick et al. 1996) in which pools with small numbers of guppies can become isolated, particularly during the dry season (Griffiths & Magurran 1997). These costs of and opportunities for inbreeding suggest that inbreeding avoidance could be advantageous in guppies, but whether these fish actively (behaviorally) avoid inbreeding is not clear.

Two studies of virgin females found no difference in their mating behaviors toward unrelated males and full-sibling males (Viken et al. 2006; Zajitschek & Brooks 2008). Similarly, in mate-choice trials comparing siblings to non-siblings, Pitcher et al. (2008) found no difference in the number of copulatory attempts made by males or in the level of pre-copulatory receptiveness of females. These studies were conducted under laboratory conditions with captive-reared fish, however, and the experimental females were virgins and/or had never been exposed to adult males before the tests, so their behavior might not reflect that of experienced fish in a more natural setting.

An ideal assessment of inbreeding avoidance would be conducted in a natural population. To that end, we examined the paternity of offspring conceived in a natural population to determine patterns of relatedness between dams and sires. We also determined the effect of pair relatedness on the number of offspring produced by free-living adult females.

METHODS

Experimental Fish

The adult guppies used in our experiment were collected in Trinidad in March 2004 from a low-predation tributary (*sensu* Endler & Houde 1995) of the Quare River (Quare 6: 696907 1181003). A low-predation site was chosen because these upland sites tend to have lower observed heterozygosity (Shaw et al. 1991, 1994) and smaller effective population sizes (Neff et al. 2008; Barson et al. 2009) and should therefore be at greater risk of inbreeding than high-predation sites. Also, sneaky copulations, which might override female choice, may be less prevalent in low-predation sites (Farr 1975; Magurran & Nowak 1991; Magurran & Seghers 1994; but see Endler & Houde 1995). This site in particular was chosen because the pools are small and can become quite isolated (drops in elevation between pools were 8–30 cm). Because each pool harbored relatively few guppies, we could collect all the adult males in a pool. We used dipnets to collect all adult males and 10–12 adult females from each of three small, interconnected pools during a single 2-h period**.** All adult males from three additional adjacent pools were also collected for assessment of paternity of males that potentially moved between pools. We returned on the two subsequent days to check for remaining males and found none.

Immediately upon collection, we separated males and females to prevent any mating after capture. Males (34 total) were euthanized in MS-222 and preserved in ethanol. Of the 34 males, 22 were collected from the same pools as the females, and 12 were collected from adjacent pools. Females (31 total) were transported to the laboratory of HR, where we collected up to six broods of offspring from each female resulting from sperm she had stored before capture.

Numbers of males and females collected from each pool are given in Table 2.1. Offspring number per female ranged from 0 (five females had no broods) to 28. All females and 229 offspring were euthanized in MS-222 and preserved in ethanol. Preserved tissue was brought to the University of Illinois at Urbana-Champaign for genetic analysis.

Genotyping

All collected adults and offspring were genotyped so that paternity could be assessed. DNA was isolated from all individuals by proteinase K extraction. We genotyped each fish at 11 polymorphic loci: AGAT11, AATG2, AGAT1, AG4 (Olendorf et al. 2004), CA, TTA (Kelly et al. 1999), Pr36, Pr40, Pr80, Pr171 (Becher et al. 2002), and G10 (Parker et al. 1998); GenBank accession numbers, number of alleles, expected and observed heterozygosities for all loci are reported in Table 2.2. All of the loci were amplified with a fluorescently labeled forward primer. PCR products were cleaned with silica membrane plates and then analyzed with an ABI Prism 3730xl Analyzer (Applied Biosystems Inc, Foster City, CA, USA) at the W. M. Keck Center for Comparative and Functional Genomics on the University of Illinois campus. We scored PCR products using Applied Biosystems GeneMapper (Applied Biosystems Inc, Foster City, CA, USA) and verified all fragment-size calls manually. Two loci, Pr40 and AG4, amplified inconsistently and were excluded from further analysis. No two adults had the same genotype, although one male was successfully genotyped at only four loci and was therefore excluded from the paternity analysis. The total number of males used for paternity analysis was 33.

Using the genotyped adults, we checked loci for deviations from Hardy–Weinberg equilibrium and for population structure between pools using the web version of GENEPOP's Fisher's statistic (Raymond & Rousset 1995). The presence of null alleles was determined by CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007).

Paternity Assignment

Paternity was assigned to offspring by CERVUS 3.0, a maximum-likelihood program that calculates logarithm-of-odds scores for candidate parents from simulations based on given allele frequencies. Simulations were run on the assumptions that 70%, 80%, and 90% of sires were captured and with a 1% genotyping error rate. Paternity was assigned to an individual when CERVUS identified the sire at the 95% confidence level or at the 80% confidence level when no allelic mismatches occurred between the offspring and its putative father. Not all offspring were assigned sires, either because their true sires were not caught or because CERVUS lacked the statistical power to assign a father with confidence. Therefore, CERVUS estimates of male and female mate number and male offspring number are underestimates.

The offspring and mothers were further analyzed with COLONY 1.2, a maximum-likelihood method of grouping full and half siblings (Wang 2004). We set the typing error rate to 1% and the additional error rate to the rate of null-alleles given by CERVUS. COLONY placed all offspring into sibling groups and therefore represents a more realistic estimate of number of mates per female than CERVUS, but COLONY did not specifically identify the males we captured as sires and cannot be used to make inferences about male reproductive or mating success. In some cases, COLONY grouped individuals as full siblings when one had been assigned paternity by CERVUS and the other had not. The previously unassigned individuals were then counted as the CERVUS father's progeny when no allelic mismatches occurred. This procedure increased the number of offspring sired per male but did not alter the number of mates assigned to each female. In the analyses, only CERVUS-assigned offspring were included, except where noted.

Relatedness and Heterozygosity Calculations

We estimated pairwise relatedness between individual adult females and males using RELATEDNESS 4.2c (Queller & Goodnight 1989) with the adult allele frequencies as the estimate of population allele frequencies. This identity-by-descent method compares the deviation from the population mean of allele frequencies within one group (or individual) to deviation in another group (or individual); the method is related to both regression- and F-statistics-based methods (Queller & Goodnight 1989). It differs from other estimates of relatedness in having a range from −1 to +1 rather than from 0 to +1. On the RELATEDNESS scale, negative values indicate that the allele frequencies of groups (or individuals) differ from the population mean in opposite directions and indicate low relatedness, whereas positive values indicate deviation from the population mean in the same direction and indicate high relatedness. The RELATEDNESS algorithm was appropriate for our data because it allowed us to combine information over all the typed loci, irrespective of their information content, and it allowed calculation of relatedness between pairs of individuals; it also has lower small-sample-size bias than do other methods (Queller & Goodnight 1989). We used the pairwise relatedness values for every possible pair of adult females and males to compare the relatedness of each female to males that they did and did not mate with (see Statistical Analysis).

Previous research (Hoffman et al. 2007) has suggested that, in some animals, female mate choice is based on heterozygosity in addition to relatedness. We therefore calculated heterozygosity for all adults that were successfully typed at four or more loci. We used standardized multilocus heterozygosity to account for different numbers of loci typed and different heterozygosity across loci (Coltman et al. 1999).

Statistical Analysis

For males, we inferred mating success based on whether they had sired at least one offspring with each female. Males with at least one assigned offspring were assigned mating success = 1 for that female; males without assigned offspring were assigned mating success = 0 for that female. We then compared the mean relatedness of females to mates and non-mates using a paired *t*-test. For each female, we calculated her mean relatedness to mates by averaging relatedness values of the female to all males with mating success = 1 for that female and relatedness to non-mates by averaging relatedness values of the female to all males with mating success = 0. A paired *t*-test then compared these two mean relatedness values for each female, under the null hypothesis that relatedness was equal in the two samples. The alternative (inbreeding avoidance) hypothesis is that relatedness is lower between females and their mates than between females and non-mates. In a separate analysis, we used a contingency table analysis to test whether females were more likely to have mated with males collected from their own pool than expected under random mating across all pools.

The association between reproductive success (the number of offspring assigned to each male–female pair) and pair relatedness was analyzed in two ways. First, we modeled reproductive success as a Poisson variable, using the canonical log link function in SAS Proc Genmod (Littell 2002), with pairwise-relatedness and pool residence as independent predictor variables (the interaction term was not significant ($p = 0.98$) and was therefore deleted from the final model). Fit of this model to the data was good (deviance = 0.91). This model assumes that each male–female pair is independent. Likelihood ratio tests with one degree of freedom were used to assess statistical significance. Because some males and females mated multiply, it was not possible to control simultaneously for non-independence of multiple matings by both males and females. When we controlled for non-independence for one sex only (e.g., females) by

nesting multiple mates within females, the data were sparse because most individuals had few mates, and the nested models did not converge. We therefore conducted a second more conservative analysis. For this analysis, we binned all data with respect to offspring number and calculated the mean relatedness of each male–female pair that produced 0, 1, 2,…9 offspring. The binning process reduced the sample size from n = 748 (all possible male–female pairs) to n = 10. The association between relatedness and offspring number was then tested with a Poisson regression model and likelihood ratio tests as above.

We assessed the relationship among individual heterozygosity, reproductive success, and mating success using non-parametric Spearman rank correlation. The reproductive-success values used for these correlations did not include offspring that were assigned sires by the full-sibling identification in COLONY, because this assignment relied on an exclusion method that might bias assignments toward more heterozygous males. Differences in variance in offspring number between females and males from the same pools were examined by means of an *F*-test of the ratio of variances in mate number in males and females.

RESULTS

Reproductive Success

Among the adults collected in Trinidad, we found no significant deviation from Hardy–Weinberg equilibrium over all loci (Fisher's method $\chi^2 = 11.35$, df = 18, p = 0.88) or genotypic differentiation between pools (Fisher's method $\chi^2 = 14.89$, df = 18, p = 0.67) and therefore no evidence for differences in relatedness among individuals between pools. Of the 229 offspring collected, 104, 105, and 128 offspring were assigned to sires by CERVUS on the assumptions

that 70%, 80%, and 90% of sires were captured, respectively. Except where noted, for subsequent analyses, the 80% assumption was used such that 22 of the 26 females that had offspring were assigned at least one mate by CERVUS.

An additional 43 offspring were assigned paternity by COLONY on the basis of full-sibling relationship with an individual with an assigned father. Table 2.3 shows the average numbers of mates and offspring for all males and females. Males and females in the three pools where both sexes were caught did not differ significantly in variance in number of offspring (*F*-test: $F_{30,21} = 0.82$, p = 0.30; Fig. 2.1a), or in variance in mate number (*F*-test: $F_{30,21} = 0.52$, p = 0.10; Fig. 2.1b).

Mating Patterns

There was no significant difference in relatedness of females to their CERVUS-assigned mates and to non-mates (relatedness to mates = −0.07 (SE = 0.053); relatedness to non-mates = +0.02 (SE = 0.006); paired *t*-test, one-tailed: $t_{21} = 1.67$, p = 0.055, see Fig. 2.2), although the trend was for females to be less related to mates than to non-mates. Thirteen matings occurred between males and females that were collected from the same pool, while 26 matings occurred between males and females collected from different pools. These numbers are not significantly different from the numbers expected if mating was random across all pools ($\chi_{[1]}^2 = 3.15$, p = 0.08). Results were similar when we assumed that 70% ($\chi_{[1]}^2 = 4.76$, p = 0.03) or 90% of sires were captured ($\chi_{[1]}^2 = 3.67$, p = 0.06). Although not significant for two of the three data sets, the trend was for more matings between animals captured from the same pool (8.3 expected under random mating), and fewer between animals collected from different pool (30.7 expected number under random mating).

Females produced more offspring with less-related males (Table 2.4; binned analysis: $\chi_{[1]}^2 = 6.1$, $p = 0.014$; Fig. 2.3). We reran the analysis of binned data after deleting the highest-influence point in the data set (that for offspring number = 5). The relationship between mean relatedness and offspring number was still significant after removal of this potential outlier ($\chi_{[1]}^2 = 12.9$, $p < 0.001$). When only those pairs that had produced offspring (offspring number >0) were included, the relationship was still highly significant ($\chi_{[1]}^2 = 9.6$, $p = 0.002$).

Females also produced more offspring with males collected from their own pools (on average 0.27, SE = 0.01, with each male) than with males collected from other pools (on average 0.10, SE = 0.03, with each male; Table 2.4). This pattern was at least partly due to the trend for more within-pool matings than between pool matings, because when the analysis was restricted only to those pairs that did mate, there was no significant difference (on average 1.20, SE = 0.15, offspring per pair when collected from same pool; 0.85, SE = 0.12, per pair when collected from different pools, $p = 0.09$). Results were similar when COLONY-assigned offspring were added and when we assumed 70% or 90% of sires were captured rather than 80% (results not shown). A non-significant trend was observed for greater variance in mate number for males than for females (*F*-test: $F_{30,21} = 0.52$, $p = 0.10$; Fig. 2.1b), but this result was probably influenced by our self-imposed constraints on the number of offspring that we reared per female.

Adult males showed a significant positive correlation between individual heterozygosity and the number of mates assigned ($\rho = 0.44$, $p = 0.01$; Fig. 2.4a). A significant positive correlation was also apparent between heterozygosity and number of offspring assigned ($\rho = 0.52$, $p = 0.003$; Fig. 2.4b). The latter correlation remained significant after exclusion of males with zero offspring ($\rho = 0.59$, $p = 0.01$). Females showed no significant correlations

between heterozygosity and number of mates assigned ($p = 0.79$) or number of offspring ($p = 0.45$; Fig. 2.4a,b).

DISCUSSION

To understand whether inbreeding avoidance could be advantageous and whether it occurs in wild populations of guppies, we evaluated mating patterns in nature. We found that mating between relatives occurred in the natural population we studied and led to substantial fitness costs in the focal population. The negative association between relatedness and offspring number (even when pairs that did not mate were excluded) suggests that pair relatedness affected fertilization probability, embryo viability, or both. Another possibility is that variation in the number of times pairs mated contributed to this association, although this seems less likely given that sperm are transferred in bundles containing more than 20 000 sperm (Magurran 2005), and ejaculate size does not appear to limit female fecundity (Pilastro et al. 2008). We cannot be confident that active behavioral inbreeding avoidance occurred, however. The association between relatedness and mating was in the direction expected if guppies actively avoid mating with relatives but was not significant ($p = 0.06$). It is possible that the lack of statistical support is due to higher sampling variance of a binomial variable (mating success) than of a Poisson variable (reproductive success) or to inherent limitations of inferring mating preferences from mating patterns. For example, unsolicited mating attempts by males might circumvent female choice. Females can be exposed to many 'sneaky' copulation attempts per day (Farr 1975; Magurran & Seghers 1994); one study estimated that a large proportion of wild-caught females carry sperm from sneak copulations (Evans et al. 2003).

Laboratory studies have not supported the idea that guppies recognize relatedness *per se* or bias reproductive behavior or reproductive effort toward non-relatives (Viken et al. 2006; Pitcher et al. 2008; Zajitschek & Brooks 2008). If inbreeding avoidance occurs, then an ability to discriminate between familiar and unfamiliar individuals, or between common and rare phenotypes, might provide the underlying sensory mechanism. Both female and male guppies can discriminate between familiar and unfamiliar conspecifics (Griffiths & Magurran 1997; Kelley et al. 1999). Females can discriminate between familiar and unfamiliar male color patterns (Farr 1980; Hughes et al. 1999; Eakley & Houde 2004; Zajitschek et al. 2006) and common and rare color patterns (Eakley & Houde 2004; Zajitschek & Brooks 2008; Hampton et al. 2009).

The decrease in offspring number with increasing pair relatedness that we observed, even after exclusion of mating patterns as a contributing factor, might be caused by biased sperm use favoring less-related males, lower fertilization success for more-related pairs, or inbreeding depression for embryo viability. Two previous laboratory studies reported no paternity bias favoring less-related males (Evans et al. 2008; Pitcher et al. 2008). Evans et al. (2008) used artificial insemination to eliminate any contribution from pre- or post-copulatory choice, whereas Pitcher et al. (2008) assessed both courtship behavior and paternity bias and found no evidence that behavior or fertilization success was biased toward less-related individuals. Together, these two studies suggest that neither sperm use nor fertilization success is affected by pair relatedness. The artificial-insemination study also suggests that relatedness does not affect embryo viability (Evans et al. 2008). In contrast, Pitcher et al. (2008) found that related pairs did produce fewer offspring, consistent with inbreeding depression for embryo viability.

The positive association we observed between male heterozygosity and both mating success and offspring number (even after dropping non-mates from the data set) is consistent with strong inbreeding depression in this population. In laboratory experiments on wild-derived guppies, several male fitness components and male ornaments exhibit inbreeding depression (Sheridan & Pomiankowski 1997; van Oosterhout et al. 2003; Mariette et al. 2006; Pitcher et al. 2008). An alternative hypothesis is that females actively choose mates with high multilocus heterozygosity (Reusch et al. 2001; Neff & Pitcher 2008; Fromhage et al. 2009). We know of no studies that have tested this hypothesis directly in guppies.

Our study provides additional insight into natural mating patterns of guppies. For example, our data illustrate that high levels of multiple mating and spatially structured populations can occur in nature. Because analyzing offspring from a limited number of broods that were fertilized with stored sperm artificially reduced the variance in offspring number and mate number, we did not calculate formal estimates of multiple paternity or reproductive skew. Nevertheless, we assigned up to five mates per female, even though the maximum number of offspring assigned paternity was 15 per female. This result supports earlier studies that concluded that guppies in nature can exhibit high levels of multiple paternity (Magurran 2005; Hain & Neff 2007; Neff et al. 2008).

We also found that females produced more offspring with males collected in their own pool than with males in adjacent pools, even though pools were only a few meters apart. We conclude that pool residence is relatively stable over short periods (interbrood intervals are approx. 25 days). The lack of significant genetic structure among pools indicates that this stability breaks down over longer periods, however. Previous work has shown that males are

more likely than females to move between pools (Reznick et al. 1996), so we attribute this breakdown primarily to between-pool movement by males.

In conclusion, the study reported here provides unique insight into natural mating patterns, and the fitness consequences of those mating patterns, in a species that is an important model in behavioral ecology. Given the already-rich literature on laboratory behavioral studies, the relative ease with which natural populations can be manipulated, and the increasing availability of molecular tools, guppies are also poised to become a preeminent model in behavioral and evolutionary genetics and genomics. Indeed, opportunities for understanding the ecology, evolution, and genetics of natural behavioral variation may be greater in this species, and in poeciliids in general, than in many other model systems.

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Table 2.1 Number of males and females typed from each pool.

Pool	Males	Females
1	8	10
2	7	11
3	7	10
4	5	0
5	3	0
6	4	0
Total	34	31

Table 2.2 Characterization of the nine *Poecilia reticulata* microsatellite loci typed in adults of the Quare 6 population.

Locus[1]	GenBank Accession number	Number of alleles	H_O	H_E
AATG2	BV097133	4 (N = 54)	0.481	0.513
AGAT1	BV097139	13 (N = 62)	0.887	0.887
AGAT11	BV097141	8 (N = 59)	0.746	0.741
CA	AF170707	3 (N = 62)	0.226	0.260
G10	AF026453	2 (N = 55)	0.255	0.224
PR171	AF467907	4 (N = 56)	0.554	0.542
PR36	AF467902	3 (N = 30)	0.217	0.196
PR80	AF467905	3 (N = 58)	0.121	0.116
TTA	AF164205	5 (N = 59)	0.424	0.414

N, number of adults successfully screened; H_O, observed heterozygosity; H_E, expected heterozygosity.

[1]Two microsatellites failed to amplify consistently: AG4 (Accession no. BV097138) and Pr40 (AF467904).

Table 2.3. Numbers of mates and offspring. Numbers are per female and per male, as determined by CERVUS 3.0 and COLONY 1.2 paternity analysis. Values are means (standard errors).

	Number of mates		Number of offspring	
	CERVUS + COLONY	COLONY	CERVUS	CERVUS + COLONY
Females*	1.290 (0.218)	1.744 (0.244)	NA**	
Females (within brood)	1.11 (0.081)	1.514 (0.086)	NA**	
Males*	1.176 (0.278)	NA	3.176 (0.915)	4.441 (1.285)

*Includes individuals that had no offspring.

**Females gave birth to 7.386 (1.357) offspring on average or 3.18 (0.257) offspring per brood. These numbers were not predicted by simulation.

Table 2.4 Results of Poisson regression of offspring number on relatedness and pool residence.

Parameter	DF	Estimate	(SE)	Deviance Ratio	$C_{[1]}^2$	P
Intercept	1	-1.299	(-0.152)	0.950	73.12	<0.0001
Relatedness	1	-0.721	(-0.261)	0.941	7.63	<0.0057
Shared pool residence	1	-0.988	(-0.198)	0.910	24.93	<0.0001

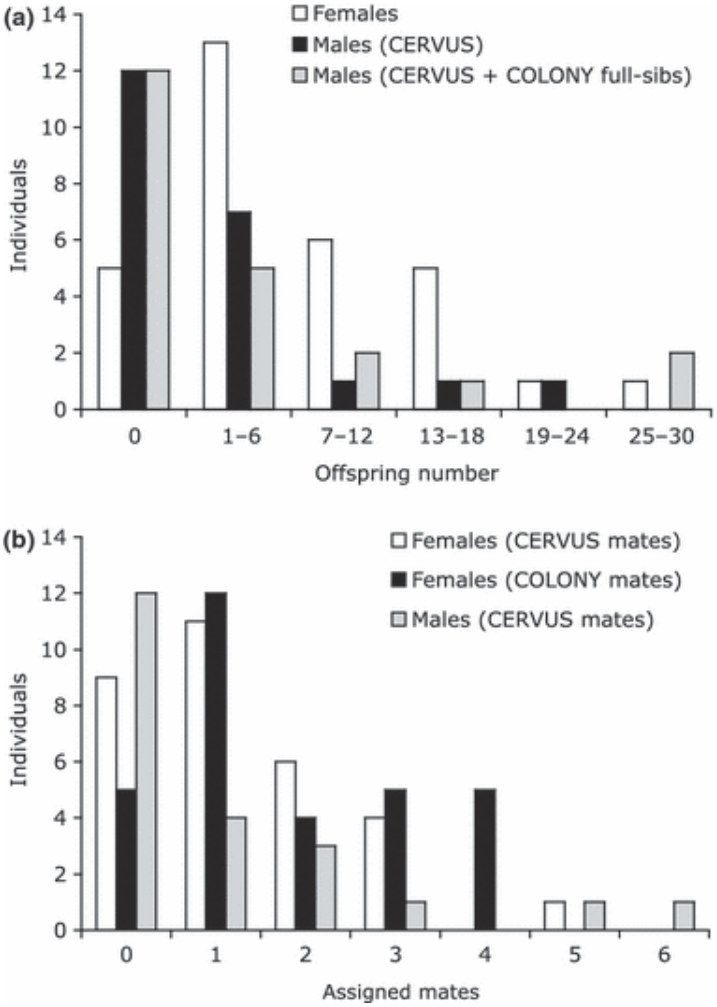

Figure 2.1. Numbers of offspring (a) and mates (b) assigned to all field-collected males and females by CERVUS 3.0 and COLONY 1.2. The CERVUS data are underestimates, as not all offspring were assigned paternity. The COLONY data estimated full- and half-sibling relationships within broods without regard to collected males and cannot therefore be used to assign mates or offspring to collected males, but in cases where COLONY identified an individual as a full sibling to a brood-mate with a CERVUS-assigned father, paternity was also assigned to that individual in the absence of allelic mismatches.

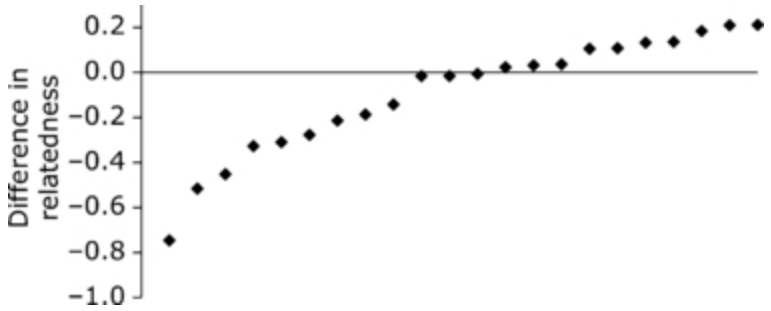

Figure 2.2. Comparison of mean relatedness between mates and non-mates for the 22 females that produced offspring that were assigned paternity with high confidence. Each diamond represents an individual female. The value on the *y*-axis indicates the mean relatedness of that female to mates minus her mean relatedness to non-mates. Thus, values <0 (below horizontal line) represent females that were less closely related to mates than to non-mates, whereas values greater than zero represent females that were more closely related, on average, to mates than to non-mates.

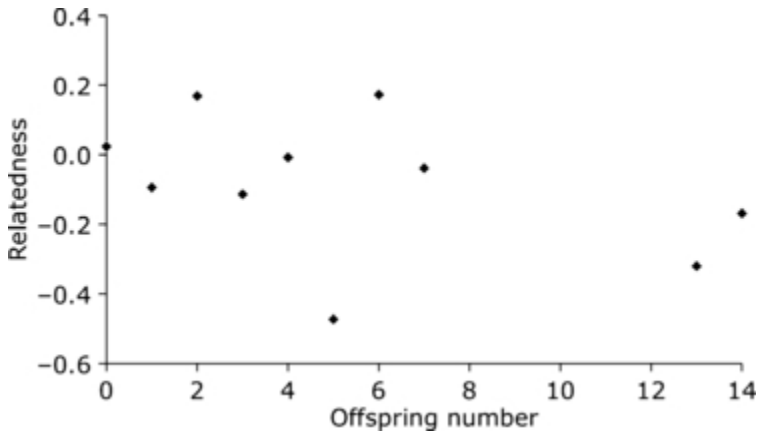

Figure 2.3. Offspring number vs. mean relatedness of all pairs assigned a given number of offspring.

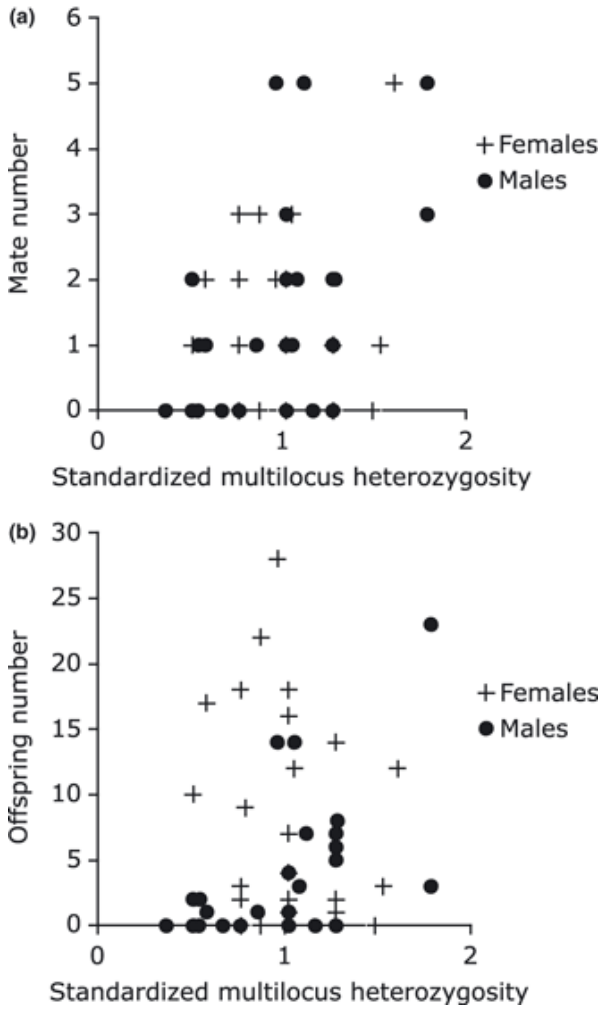

Figure 2.4. Male standardized multilocus heterozygosity and (a) number of mates assigned by CERVUS and (b) number of offspring assigned by CERVUS. Male correlation is significant even without the outlier of 23 offspring.

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CHAPTER 3

MELANIN, CAROTENOID, AND PTERIN ORNAMENTS IN THE BLUEFIN KILLIFISH *LUCANIA GOODEI*

ABSTRACT

Male bluefin killifish (*Lucania goodei*) exhibit extensive color variation in their fins, but the role, if any, of this variation has not yet been determined. We collected males from multiple populations across Florida and used absorption spectroscopy to identify the pigments responsible for the fin coloration. We determined that orange coloration in the caudal fin is caused by a carotenoid pigment. In contrast, color in the anal fin is either pterin based (yellow and red) or structural (blue) with a melanic fin border. As these colors have different developmental origins, the potential for complex signaling in this species is high. Using a behavioral assay designed to measure dominance, we sought to determine the informational content of each pigment. Black melanic markings on the anal fin are positively correlated with dominance and mating success. Aggression is greater between males of similar sized melanic stripes, indicating that they function as badges of status in territorial interactions with other males. In keeping with their dietary origin, caudal carotenoid levels are correlated with condition but do not influence dominance interactions. However, this highly labile ornament predicts parasite infection and spawning success, suggesting a role in intersexual selection, with caudal carotenoid as a signal of health to potential mates. Similarly, pterin pigmentation in the anal fin, while not related to

dominance, is related to overall spawning levels and parasite infection, suggesting that pterin pigmentation may also signal immune status.

INTRODUCTION

The informational content of animal coloration has long captivated scientists. In cases where color differs across individuals, scientists have identified examples of honest (or dishonest) indicators of condition, advertisements for good genes, badges of status, or examples of runaway evolution (reviewed in Andersson 1994; Searcy and Nowicki 2005). Often times, animals differ in multiple aspects of coloration, and in these cases, the obvious question is why multiple ornaments have evolved where perhaps one would do. Møller and Pomiankowski (1993) examined why birds have multiple ornaments and put forth three hypotheses: (a) the multiple message hypothesis, where each ornament signals a different quality of the animal, (b) the redundant signal hypothesis, where the ornaments combine to give a more obvious signal, and finally (c) the unreliable signal hypothesis, where signals are unreliable and exist in multiples because they are cheap to produce and may have a small benefit. Møller and Pomiankowski focused on intersexual signals when they developed their hypotheses, but Andersson et al. (2002), pointed out that intrasexual selection may also produce signals. They proposed the multiple receiver hypothesis, in which multiple ornaments exist as signals to different receivers.

When examining specific cases, the developmental origins of each ornament may give valuable hints as to which hypothesis explains the origin of the multiple ornaments/signals. If all ornaments are produced the same way, then they may be combining into one overall "redundant

signal." However, if each signal has a different developmental pathway, each may be responsive to different aspects of the animal and combine to send "multiple messages" to "multiple receivers."

Scientists have developed an extensive literature examining the informational content of signals composed of carotenoids and melanins that is predicated in part upon the physiology behind their development (McGraw 2005; Searcy and Nowicki 2005). For example, carotenoid-derived ornaments are frequently assumed to be honest signals based on the fact that animals cannot synthesize carotenoids *de novo* and must obtain them via diet (Olson and Owens 1998). At their simplest then, carotenoid signals convey information to potential mates about foraging ability or condition, especially in carotenoid-limited environments (Brush and Power 1976; Kodric-Brown 1989; Hill 1992; Hill and Montgomerie 1994; Grether 2000). Carotenoids can also be antioxidants (McGraw 2005), and their role in immune function suggests that carotenoid-based signals may indicate current health or parasite infection status (Hamilton and Zuk 1982) or predict the ability to fight off future infection (Lozano 1994). In fact, an extensive literature exists that links carotenoid levels to immunocompetence in birds and fish (Milinski and Bakker 1990; Houde and Torio 1992; Brawner et al. 2000), and multiple studies have suggested that females have keyed into the utility of carotenoid-based ornaments to the effect that they do influence female mate choice (Semler 1971; Houde 1987; Milinski and Bakker 1990; Hill 1991; Bakker and Mundwiler 1994; Hill et al. 1999; Hill et al. 2002).

The proposed signaling functions of diet-derived carotenoid ornaments stand in contrast to those the other well-studied pigment, melanin. Melanin-based color patterns are synthesized *de novo* by the organism and are thought to be cheap signals to manufacture (Gonzalez et al. 1999; McGraw et al. 2002). Thus, even low-condition individuals should be able to manufacture

them. However, these supposedly inexpensive and irrelevant signals are used quite often in socially critical dominance interactions (e.g. Jarvi et al. 1987; Senar et al. 1993; Horth 2003). These cheaply produced, long lasting signals of dominance (or more aptly fighting ability) have been labeled "badges of status." It has been hypothesized that the honesty of these signals is maintained because of the high cost of lying in intrasexual interactions, where "cheaters" are subject to increased aggression (Tibbetts and Dale 2004; Searcy and Nowicki 2005)

The relationship between pigment origin and function is not always clear, however. In addition to dominance interactions, melanin has also been associated with immune function and oxidative stress (Fitze and Richner 2002; McGraw 2005; Galvan and Alonso-Alvarez 2009), and carotenoid signals sometimes function as badges of status (Pryke et al. 2002; Pryke and Andersson 2003). In addition, the qualities of lesser known pigments, such as pterins, are even more unclear. Pterins, which like melanins can be synthesized *de novo*, have also been identified as antioxidants (McGraw 2005). However, only a very limited number of studies in penguins and lizards have thus-far linked pterins with condition (McGraw et al. 2009; Weiss et al. 2012) or immunocompetance (Nolan et al. 2006).

Here, we use the bluefin killifish (*Lucania goodei*) to examine whether multiple ornaments convey redundant messages, multiple messages, or nothing at all. The bluefin killifish is a small freshwater fish found in the southeast United States. Males are territorial. Females visit multiple males, are courted, and allocate their eggs across several males throughout the breeding season (Breder and Rosen 1966; Fuller 2001). While females are largely colorless, male fins exhibit color variation in three separate areas (Figure 3.1). First, males have an orange caudal fin that can vary widely in amount of orange present. Second, their anal fin varies both

in color morph (red, yellow, or blue) and saturation of color. Third, the anal fin also varies in the extent of black outlining the distal end of the fin.

The multiple aspects of fin ornamentation make the killifish an ideal system to study multiple signals. The informational content of the orange coloration on the caudal fin and the black coloration on the anal fin has not been examined at all, and the function of the anal fin color morphs has remained elusive in the studies that have tried to address it. The anal polymorphism is unlinked to any obvious behavioral types (McGhee et al. 2007; McGhee and Travis 2010), and while a small female preference for males with red over yellow anal fins has been detected in some studies (Fuller and Johnson 2009; Fuller and Noa 2010), others have failed to show this pattern (McGhee et al. 2007; McGhee and Travis 2010). While little is known about the fitness correlates of these colors, the genetic/environmental control of the anal fin color morphs have been examined in some detail. The red/yellow anal fin polymorphism is largely genetically determined with a single locus of large effect in which yellow is dominant to red; blue is orthogonal to the red/yellow polymorphism and its prevalence is affected by both genetics and lighting environment (Fuller and Travis 2004).

The goals of this paper were to (1) determine which pigments are used in *L. goodei* anal and caudal fins and to (2) determine whether these pigments predict male dominance, male spawning success, or overall condition, which would indicate signal function. In our first study, we determined the pigment classes responsible for the red, yellow, blue, and orange coloration on the fish's anal and caudal fins using absorption spectroscopy on individuals from multiple populations across Florida. In our second study, we performed behavioral observations where we allowed two males to repeatedly compete over a female over four days and measured male dominance and courting behaviors. By monitoring male-male-female interactions in behavioral

trials and quantifying coloration and pigment levels in the anal and caudal fins, we were able to determine the informational content of these fin ornaments. Following the behavioral trials, we determined male body condition and macroparasite loads. Hence, this study allowed us to elucidate the relationships between pigment content, male behaviors, and male health.

METHODS

Pigment Identification

The fish used to identify pigment class were collected with dipnets and seines from five populations in Florida: Upper Bridge and St. Marks Refuge in the Wakulla drainage (11 and 8 males respectively); Delks Bluff in the Oklawaha drainage (11 males); Wacissa Springs in the Aucilla drainage (8 males); and 26-Mile Bend in the Everglades (8 males). Fish were held without food in water collected from their site of origin and were euthanized within five days of collection. Fins were removed and frozen until pigment could be analyzed. To identify pigments, individual anal and caudal fins were ground in 1 ml 1% NH_4OH using a mortar and pestle. One ml of a 1:1 hexane:tert-butyl methyl ether solvent was added to elute carotenoids. The absorption spectra of both solvent layers were examined to determine pigment class. Pterin pigment was identified by a strong absorption in the UV in the NH_4OH layer, and carotenoid was identified by a shouldered peak in the hexane:tert-butyl methyl ether solvent. Melanic and structural coloration was identified by an inability to go into solution.

Behavioral trials

The fish used in behavioral trials were collected with seines and dip nets from the Upper Bridge of the Wakulla River, Wakulla County, Florida population near Tallahassee, Florida.

Fish were housed in a communal stock tank (~300 liters) located in a climate-controlled greenhouse at the University of Illinois with supplemental light from Xenon lamps (which supplement the ultraviolet portion of the spectrum) providing a 14h light, 10h dark schedule. Fish were fed frozen adult *Artemia* and flake food. Fish also had access to naturally occurring invertebrates and algae growing in the stock tank.

Fifty behavioral trials were conducted in 2010. Because blue morphs are rare in this population, only red and yellow morph males were utilized in the behavioral trials. For each set of trials, adult male fish were selected at random and isolated physically and visually from each other in individual five-gallon (19 liter) tanks. After three days of isolation, the males were randomly paired and anesthetized with a 0.025% MS-222 solution in the late afternoon/evening. Each pair was moved to a petri dish filled with a small amount of water, placed against a white background with a color standard, and a picture was taken of their left sides. Each pair was then placed in a thirty gallon (114 liter) tank with a female from their cattle tank and five yarn mops (three floating, two sinking) that the fish use as shelter and a spawning substrate. During the following four mornings, behavioral observations were taken for two non-consecutive 20-minute bouts between 0700 and 1100 each day.

Individual males were identified by size, shape, and pigmentation during the observations. Counts of male-male aggression (fin flares, chases, sigmoids, and attacks resulting in physical contact) for each individual male were recorded. Male-female interactions (fin flares, physical attacks, chases, and courting bouts, i.e. head flicks directed at the female) were also noted. Spawning was recorded for a male when the male was observed in contact with a female while in the yarn mops and eggs were subsequently found in the tank. Circle fights, where both males attack each other repeatedly while circling each other, and chases involving

both males and the female, were also recorded for the tank, although these behaviors were not assigned to a particular male. At the end of each morning's observations, mops were searched for eggs, and the eggs were discarded.

Two separate observations over each of the four days of the trial yielded a total of eight observations for each pair. Each observation was evaluated separately, and the dominant individual was determined for that observation. The individual was scored as dominant for that observation if he initiated more aggressive interactions than his partner. For some observational bouts (19 in 400), neither male performed any behaviors, and in these cases, neither male was recorded as dominant for that observation. Thus, each male could be scored as dominant up to eight times, and this provided their overall dominance score. The male with the higher dominance score was labeled as the overall dominant male of the pair. There were no ties in dominance score observed across our 50 trials.

Pigmentation and color

Following the four days' observations, the male pairs were euthanized in MS-222. The standard length and wet weight of each male were recorded, and the males were again photographed in their pairs. The anal and caudal fins were removed from the fish and stored at -80C. The fish were placed in ethanol and also stored at -80C. Males were later examined under a dissecting scope for acanthocephalan macro-parasites in their body cavity.

To measure carotenoid pigment content in the caudal fin, fins were ground in 1 ml 1% NH_4OH using a mortar and pestle. The pigment was then transferred to 1 ml of a 1:1 hexane:tert-butyl methyl ether solvent via vortexing and measured on a spectrophotometer. Maximum absorption (445 nm) was used as an indicator of quantity of carotenoid. To determine

pterin pigment content in the anal fin, the fin was ground in 400 µl of 1% NH_4OH, and absorption was measured at 398 nm (yellow) and 498 nm (red) in a spectrophotometer.

In addition to measuring carotenoid coloration via pigment extraction, we also used digital photography to assess male coloration. The advantage of photography is that coloration can be assessed both before and after the behavioral trials. Photographs of the fish from before and after the behavioral trials were used to quantify change in caudal fin coloration over the course of the trial. Picture light and color levels were standardized using the PicoColor 4.5 Photoshop Plug-in, which used the color standard in the image for calibration. In ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/), the caudal fin was outlined using the freehand selection tool. All colors in the caudal fin besides orange were removed using the "Threshold_Color" plug-in, and the number of pixels was counted to measure amount of orange. The number of pixels was adjusted by using a size standard to account for minor differences in magnification between pictures. The difference in pixel number between pictures taken before and after the trials was used to indicate a change in amount of orange over the course of the trial. We also used digital photographs to assess black coloration (i.e. melanin) on the male anal fins before and after the behavioral trials. Using imageJ, the distal ends of the anal fins, which contain a black band, were isolated from both males in the picture using the freehand selection tool. The image was converted to black and white by using the adjust threshold function and selecting black and white threshold color. The histogram tool was used to count the number of black pixels on each anal fin.

Using these methods, we obtained several measures of pigmentation: black anal coloration (pictures both before and after the trial); orange on the caudal fin (levels of carotenoid pigmentation assessed via absorbance at 445 nm and orange coloration assessed via digital

photography both before and after the trials); and anal red/yellow pigmentation (yellow pterin measured as absorbance at 398 nm, red pterin measured as absorbance at 498 nm, total pterin levels as those absorption levels summed, and color morphs as assigned by AJ). Table 3.1 lists and defines the color variables measured for each fish. We used Pearsons correlations to determine if the pigments were correlated with each other. When necessary, we also obtained residual pigment values by regressing overall pigment levels on standard length (hereafter length). To determine if the different morphs differed in their amount of each pigment, we used general linear models (proc glm) to compare them.

Our first goal was to determine which elements are correlated with male dominance and spawning success. Because we were able to assign male dominance for every pair, we used simple paired t-tests to ask which elements varied between dominant and subdominant pair members. We also looked at which pigments affected overall dominance score. In this case we used generalized linear models (proc genmod) where the distribution of the data was modeled as binomial with logit link function. Because the observations were not independent (i.e. the behavior/score of one male depended on the behavior of another), we included male pair as a repeated factor in the analysis. We performed a similar analysis on spawning and other behaviors where the model considered the total number of spawns a male performed given the total number of spawns observed for both males as a function of male coloration, where male pair was treated as a repeated factor. When measuring overall counts of spawning, we also used generalized linear models with a negative binomial distribution due to the high number of zeros and male pair as a repeated factor. When considering other behaviors, we used the same process with a normal distribution.

Our second goal was to determine the relationship between male health and pigmentation. We measured body condition as the residuals of the regression of $\log_{10}$ of weight on $\log_{10}$ of length (Bolger and Connoly 1989) and used Pearsons correlations to determine if body condition was related to pigmentation. As another measure of health, we used infection with acanthocephalan parasites. Infection was either binary (parasitized or not), in which case we used logistic regression (proc logistic), or we modeled the number of parasites each individual was infected with (parasite load) as a generalized linear model (proc genmod) using a negative binomial distribution to account for the high number of zeros.

RESULTS

Pigment identification

The orange pigment extracted from the caudal fins across all the populations had an absorption spectra characteristic of a carotenoid (McGraw et al. 2005) (Figure 3.2). In contrast, the yellow and red pigments extracted from the anal fins of the fish had absorption spectra characteristic of pterins. Yellow morph males had a single pterin peak of 398 nm, indicating the presence of a yellow pterin while red morph males had a red peak at 498 nm in addition to the yellow peak at 398 nm, indicating that red-morph males produce two separate pterin pigments (Figure 3.3). No pigment was detected in blue males, where the absorption spectra matched colorless females (Figure 3.3), indicating that blue coloration was due to structural reflectance properties rather than pigmentation. Black coloration on the anal fin did not go into solution, indicating that it was melanin (McGraw et al. 2005).

Relationships among pigments

Pigmentation was examined in greater depth using the fish in our behavioral study. As we had many measures of pigmentation, we have defined these measurements and summarized our results in Table 3.1 for clarity. In addition, Table 3.2 defines the variables for which we measured pigment associations.

In these fish, the regressions of carotenoid and total pterin levels (red and yellow absorption summed) on standard length of the fish were highly significant (carotenoid: T_1 = 10.59, $P < 0.0001$; total pterin: $T_1 = 5.49$, $P < 0.0001$), with bigger fish having higher values, though this was not the case for melanin ($P = 0.0947$). More specifically, in regard to pterin pigmentation, all males had yellow pterin pigmentation, which correlated with length ($r = 0.52$, $P < 0.0001$), but red pterin pigmentation levels did not correlate with length ($P = 0.13$), presumably because the possession of red was limited to red morphs only.

We sought to determine if the fish pigments were independent of each other after correcting for size of the fish by using length as a partial correlate. The amount of black on the anal fin and total pterin on the anal fin were correlated ($r = 0.21$, $P = 0.042$), probably as a result of differential fin sizes relative to length. However, caudal carotenoid levels were not correlated with either of the other classes of pigments (melanin: $r = 0.11$, $P = 0.29$; total pterin: $r = 0.045$, $P = 0.66$). Thus, caudal pigmentation is completely independent of anal pigmentation. We also checked if the red and yellow morphs had significantly different amounts of carotenoid and melanin. We found that there was no difference in the amount of melanin ($black_{post\text{-}trial}$ $F_{1,98}$ = 0.27, $P = 0.6$; $black_{pre\text{-}trial}$ $F_{1,98} = 1.38$ $P = 0.24$) or carotenoid pigment ($F_{1,98} = 2.72$, $P = 0.1$). There was also no difference between the morphs in amount of yellow pterin ($F_{1,48} = 0.01$, $P = 0.93$) while, as expected, morphs visually categorized as red had significantly more red pterin pigment $F_{1,48} = 13.54$, $P = 0.0006$)

Multiple measures of the same color element were correlated with one another. The photographic pre-trial and post-trial measures were highly correlated for both caudal orange ($r = 0.77$, $P < 0.0001$) and anal black ($r = 0.56$, $P < 0.0001$). The photographic measures of orange were also highly correlated with carotenoid pigment measures ($orange_{pre\text{-}trial}$ and carotenoid pigment: $r = 0.53$ $P < 0.0001$; $orange_{post\text{-}trial}$ and carotenoid pigment: $r = 0.58$, $P < 0.0001$), indicating that the photographs captured the degree of orange pigmentation well.

Pigments as predictors of dominance

The numbers of aggressive and courting behaviors strongly differed between males identified as dominant and subdominant (Table 3.2), yielding a clear differentiation between dominant and subdominant individual dominance scores (Figure 3.4). Not only was the behavior of individuals labeled dominant and subdominant different, but dominant males spawned more than subdomiant males (57 total spawning events observed, $X_1^2 = 9.31$, $P = 0.0023$), indicating that assigned dominance directly affected fitness. Dominant males differed only slightly in length and weight from subdominant males, and these differences were not significant (paired t-test, length: $T_{49} = 1.82$, $P = 0.076$; weight: $T_{49} = 1.75$, $P = 0.086$) (Table 3.3).

We looked for associations between each pigment class and dominance. Melanin was a strong predictor of dominance. Dominant individuals had significantly more melanin on the distal portion of their anal fin in photographs taken from both before the trial began (paired t-test, $T_{49} = 2.64$, $P = 0.01$) and at the conclusion of the observations (paired t-test, $T_{49} = 2.89$, $P = 0.0057$) (Figure 3.5). This relationship remained when using residuals that were corrected for the marginally non-significant effect of length on black (size-corrected $black_{pre\text{-}trial}$: paired t-test, $T_{49} = 2.33$, $P = 0.024$; size-corrected $black_{post\text{-}trial}$: paired t-test, $T_{49} = 2.75$, $P = 0.0084$). The raw and size-corrected amounts of black were also significant when predicting the overall dominance

score out of eight observations ($black_{pre\text{-}trial}$: $X_1^2 = 4.41$, P = 0.036; $black_{post\text{-}trial}$: $X_1^2 = 4.93$, P = 0.024, size-corrected $black_{pre\text{-}trial}$: $X_1^2 = 4.18$, P = 0.041; size-corrected $black_{post\text{-}trial}$: $X_1^2 = 4.93$, P = 0.026).

Overall aggression between a pair of males was highest when the two males had similar melanin levels. There was a significant positive correlation between percent similarity in anal fin melanin and total male-male aggression (spearman correlation, r = 0.59, P < 0.0001) (Figure 3.6). There was no correlation between percent similarity in anal fin melanin and total male-female aggression (spearman correlation, r = 0.01, P = 0.9).

Anal fin pterin pigmentation was not related to dominance. The effect of anal fin color morph on dominance was not significant. Of the 50 trials recorded, 25 were yellow-yellow, 5 were red-red, and 20 were red-yellow male pairs. Among the 20 red-yellow pairs, yellow was dominant over red 13 times, but this was not significantly different than what was expected by chance (binomial test, two-tailed, P = 0.2632). Though total pterin pigment in the anal fin was correlated with the amount of melanin, total pterin did not predict dominance either in raw amount (paired t-test $T_{49} = 1.65$ P = 0.10) or after using residuals corrected for length ($T_{49} = 1.05$ P = 0.30). Yellow males did tend to exhibit more aggressive behaviors (flares, chases, attacks, and sigmoids) towards their tankmate than red males ($X_1^2 = 3.81$, P = 0.051), and this tendency was especially pronounced after including similarity in anal fin melanin in the model (morph: $X_1^2 = 5.33$, P = 0.021; anal fin melanin similarity: $X_1^2 = 11.56$, P = 0.0007).

Carotenoid pigmentation also did not reflect dominance. There was no difference in carotenoid levels between dominant and subdominant fish in carotenoid pigment (paired t-test, $T_{49} = 1.67$, P = 0.10) or size-corrected carotenoid (paired t-test, $T_{49} = 0.77$, P = 0.44). Interestingly, the carotenoid pigment in the caudal fin was notably labile. Males placed in

isolation lost orange pigmentation, and pictures taken before the behavioral trials reflected this loss. However, during the ~15 hours between being placed in the observation tank and the initiation of behavioral observations the following morning, carotenoid levels rapidly increased (Johnson, personal observation). This plasticity was reflected in a significant increase in caudal fin pigmentation based on caudal $orange_{pre\text{-}trial}$ and $orange_{post\text{-}trial}$ (paired t-test, $T_{99} = 6.64$, $P < 0.0001$). Dominant males did not have a larger percent increase in the amount of caudal orange than subdominant males either as a group (t-test, $T_{98} = 0.08$, $P = 0.9$) or correcting for trial partner (paired t-test, $T_{49} = 0.09$, $P = 0.93$).

Pigments as predictors of spawning success

In addition to strongly predicting dominance, the amount of melanin also predicted spawning success out of the total number of spawns observed within a pair ($black_{pre\text{-}trial}$ $X_1^2 = 6.7$, $P = 0.0096$; $black_{post\text{-}trial}$ $X_1^2 = 6.7$, $P = 0.0096$, size-corrected $black_{post\text{-}trial}$ $X_1^2 = 6.26$, $P = 0.012$). Males with higher levels of carotenoid also had higher spawning success ($X^2_1 = 4.24$, $P = 0.0395$), although this relationship did not remain significant when using size-corrected carotenoid ($P = 0.0904$). There was no difference between anal fin morphs in spawning success ($X^2_1 = 1.03$, $P = 0.3$). Total pterin was not related to proportion of successful spawns either before ($X^2_1 = 2.98$, $P = 0.084$) or after correcting for length ($X^2_1 = 1.64$, $P = 0.2$).

As another measure of spawning, we also considered whether the number of spawning events observed overall (rather than proportion within each male pair) was related to pigmentation. In this case, more melanic males did not have more spawning events attributed to them ($black_{post\text{-}trial}$ X^2_1, $= 3.33$, $P = 0.068$; size-corrected $black_{post\text{-}trial}$ X^2_1, $= 3.1$, $P = 0.078$), nor did males with more carotenoid have a higher number (carotenoid X^2_1, $= 0.76$, $P = 0.38$; size-corrected carotenoid X^2_1, $= 0.05$, $P = 0.82$). However, males with higher total pterin did have a

higher number of spawning events (total pterin X^2_1, = 6.00, P = 0.014; size-corrected total pterin X^2_1, = 6.22, P = 0.013).

Pigments as predictors of health

We used two measures of male health. We measured body condition as the residuals of $\log_{10}$ of mass on $\log_{10}$ of length. None of the measures of melanin correlated with condition and neither did total pterin. However, all measures of caudal carotenoid positively correlated with body condition except those from the pre-trial photographs (carotenoid: r = 0.21, P = 0.040; size-corrected carotenoid: r = 0.29, P = 0.0036; $orange_{post\text{-}trial}$: r = 0.24, P = 0.016; size-corrected $orange_{post\text{-}trial}$: r = 0.29, P = 0.0037) (Figure 3.7).

As another measure of male health, we looked at infection with acanthocephalan parasites. There was no relationship between any measure of melanin and infection, which was reflected in the fact that dominant males were no more likely to be infected than subdominant males (P = 0.92). However, pterin pigment levels had an interaction with length that predicted acanthocephalan infection (logistic regression, pterin: X^2_1 = 7.67, P = 0.006, length: X^2_1 = 9.69, P = 0.0018, total pterin*length: X^2_1 = 7.71, P = 0.0055) (Figure 3.8a). Caudal pigmentation also predicted infection (logistic regression, carotenoid: X^2_1 = 3.24, P = 0.072, length: X^2_1 = 8.83, P = 0.003, carotenoid*length: X^2_1 = 3.9, P = 0.048; $orange_{post\text{-}trial}$: X^2_1 = 4.30, P = 0.038, length: X^2_1 = 13.1, P = 0.0003, $orange_{post\text{-}trial}$*length: X^2_1 = 5.56, P = 0.018, $orange_{pre\text{-}trial}$: X^2_1 = 4.28, P = 0.039, length: X^2_1 = 7.56, P = 0.006) (Figure 3.8b). In both of these cases, larger males that were infected had less pigment than expected. The same results were significant when predicting parasite load (total pterin: X^2_1 = 5.51, P = 0.019, length: X^2_1 = 7.89, P = 0.005, total pterin*length: X^2_1 = 5.35, P = 0.027; carotenoid: X^2_1 = 4.88, P = 0.027, length: X^2_1 = 9.23, P = 0.002, carotenoid*length: X^2_1 = 5.27, P = 0.021; $orange_{post\text{-}trial}$: X^2_1 = 4.16, P = 0.04, length: X^2_1 =

13.71, P = 0.0002, $orange_{post\text{-}trial}$*length: $X^2_1 = 5.58$, P = 0.018, $orange_{pre\text{-}trial}$: $X^2_1 = 6.25$, P = 0.012, length: $X^2_1 = 9.15$, P = 0.0025).

DISCUSSION

Our results show that coloration in the bluefin killifish (*Lucania goodei*) originates from multiple sources. Coloration in the anal fin exploits melanin, pterin, and structural elements; blue anal fin morphs utilize blue structural coloration, yellow morphs utilize a yellow pterin pigment, and red morphs utilize both a red and yellow pterin pigment. In addition, the anal fins are accented by a melanic border. The orange color variation in the caudal fin is independent of the anal fin and caused by varying amounts of a carotenoid. This pattern of pigment utilization is similar in all the populations we have examined thus far. The co-occurrence of these multiple pigments allows us to examine the purpose for which they might have evolved. While our behavioral study does not examine blue structural coloration due to its rarity in our focal population, we were able to evaluate the potential significance of the melanin, carotenoid, and pterin-derived ornaments in the killifish.

Melanin

We demonstrate clearly that melanin is implicated in dominance interactions in the bluefin killifish; the more melanic of the two males in each behavioral trial was more likely to be dominant (Figure 3.5) and obtained a higher proportion of the spawns with the female. In a natural setting, males must first compete with each other to establish territories before courting females. Thus, establishing dominance quickly and efficiently is likely highly important in these fish, and the melanic border may serve as a signal to facilitate these male-male interactions.

It is possible that the melanic fin border, while correlated with fighting ability, has no actual effect on receiver behavior and is not a signal to other males. Rather, a link between melanism and aggression could be induced by pleiotropic effects of melanocortins. For example, melanocortins bind not just to the melanocortin 1 receptor in the skin, but also have weak affinity for the four other melanocortin receptors in other organs throughout the body. It has been suggested that pleiotropic binding effects could have substantially alter behavior (reviewed in Ducrest et al. 2008). However, we show that aggressive interactions between male pairs are increased when the ornaments are of similar size, while having no effect on aggressive actions towards females. This targeted escalation suggests that the ornament is functioning as a "badge of status" (Hurd 1997).

If, as our results indicate, it is highly advantageous to be a dominant male, what keeps males from evolving dishonest badges to improve their dominance status? Melanin is easily synthesized (Gonzalez et al. 1999; McGraw et al. 2002), and as a result, the honesty of this signal is most likely not derived from the cost of manufacturing it. However, honesty in badges may be maintained by associated costs. For example, testosterone has been proposed to increase badge size in birds (Evans et al. 2000; Buchanan et al. 2001) while lowering immune function (Folstad and Karter 1992). This would make the badge an immunocompetance handicap that only high quality males could afford. However, our experiments found no link between badge size and condition or immune function in killifish, suggesting that this is not the case. Rather, it seems more likely that honesty in the killifish badge is maintained by "social control" via the consequences of deception (Rohwer 1977). Interestingly, it has been suggested that in systems like this one, where aggression is increased among individuals with similar sized badges, social

control is an especially effective method of maintaining honesty (Hurd 1997). This is because males who fake a larger badge suffer more from conflicts with high status males.

Carotenoid

The caudal carotenoid-based ornament appears to signal different information than the anal melanic stripe. Though the ornament was not associated with dominance, males with higher levels of carotenoids did obtain a higher proportion of the spawnings in their tank. Carotenoid levels were positively correlated with body condition (Figure 3.7), a relationship one might expect given the pigment's association with diet. In addition, parasitized males had lower levels of carotenoid pigmentation (Figure 3.8b).

This pattern might be explained by female choice. In a natural setting, a female evaluates multiple males and spreads her eggs across them. The signals males use to advertise themselves to females might be distinct from those they use in dominance interactions with other males. We found that carotenoid levels are highly associated with body condition. Females might therefore use caudal carotenoid levels to evaluate male condition. As carotenoid levels in males are directly tied to diet, and therefore presumably honest, mate choice decisions by females may strongly factor in the evolution of this signal. The fact that carotenoid coloration is reduced in isolation but increased in the presence of other fish (including females) suggests that carotenoids function in signaling - perhaps to females.

Pterins

No relationships between dominance and pterin morph or total pterin levels were found. However, pterin pigmentation was related to parasite levels (Figure 3.8a). Larger males that were infected with acanthocephalan parasites had less anal fin pigmentation than males that were uninfected. Pterin pigmentation is just beginning to be studied in organisms. While, like

melanins, pterins are presumed to be easy pigments to manufacture, studies are beginning to link pterins to immune function due to their potential antioxidant properties (McGraw 2005). Males with higher levels of pterins did spawn at higher levels overall. These results suggest that females may use pterin pigmentation in addition to carotenoid pigmentation to evaluate the immunocompetance of potential mates. This research is an important step in beginning to understand the functions of these pigments.

Conclusions

Our results clearly show that the melanic anal fin border is related to dominance in *Lucania goodei*. As our study was primarily designed to assess dominance, our results in regard to female choice are less robust. However, it appears that females also prefer males with high carotenoid and pterin levels, perhaps because they serve as signals of condition and immunocompetence. Our results in this regard are conservative because the focal animals had been living for some time in captivity under an abundant diet and without exposure to parasites (i.e. parasites were presumably obtained in the field). This suggests that carotenoid and pterin signals may be more important for intersexual interactions than our study could convey. The relationship between dominance and spawning success might be inflated in the lab because a single male can monopolize an entire tank (McGhee et al. 2007; McGhee and Travis 2010). However, in nature females can more freely travel between territories, and in this case, females may use caudal carotenoid and pterin content to assess the condition or immunocompetance of potential mates as they lay their eggs across multiple males. To truly interpret the meanings behind these various pigments would require manipulation of the ornaments (Sheldon and Verhulst 1996). Therefore cautious interpretation of these results is required.

Our work is the first to simultaneously examine the potential informational content in melanin, carotenoid, and pterin-based ornaments. Considered in total, our work lends support to the multiple receivers hypothesis. Melanin in these fish is a badge of status that signals dominance to other males during territorial conflicts. Caudal carotenoid levels signal condition to females and perhaps imunocompetence. Anal pterin levels may also signal imunocompetance to females. Each of these signals has a different developmental origin which dictates its signal function.

Table 3.1. Variables related to coloration, their definitions, and major results.

	Variable	Definition	Results
	Black$_{pre\text{-}trial}$	Number of black pixels in digital image of anal fin; pictures taken prior to behavioral trials	Positively correlated with dominance and dominance score.
	Black$_{post\text{-}trial}$	Number of black pixels in digital image of anal fin; pictures taken after behavioral trials	Positively correlated with dominance and dominance score. Predicts spawning success.
	Size-corrected black$_{pre\text{-}trial}$	Residuals from the regression of black$_{pre\text{-}trial}$ on standard length	Positively correlated with dominance and dominance score.
	Size-corrected black$_{post\text{-}trial}$	Residuals from the regression of black$_{post\text{-}trial}$ on standard length	Positively correlated with dominance and dominance score. Predicts spawning success.
	Similarity in anal fin melanin	Black$_{post\text{-}trial}$ of less melanic fish divided by black$_{post\text{-}trial}$ of more melanic trial partner	Positively correlated with total male-male aggression.
	Caudal carotenoid pigment	Absorbance of the caudal fin at 445 nm	Predicts spawning success. Correlated with body condition. Predicts parasite infection status and parasite load.
	Size-corrected carotenoid pigment	Residuals from the regression of caudal carotenoid pigment on standard length	Positively correlate with body condition.
	Orange$_{pre\text{-}trial}$	Number of orange pixels in a digital image of caudal fin; pictures taken prior to behavioral trials	Predicts parasite infection status and parasite load.
	Orange$_{post\text{-}trial}$	Number of orange pixels in a digital image of caudal fin; pictures taken after behavioral trials	Positively correlated with condition. Predicts parasite infection status and parasite load.
	Total pterin pigment	Summed absorbance of anal fin at 398 nm and 498 nm, representing sum of yellow and red pterin pigments.	Predicts spawning events. Predicts infection status and parasite load.

Table 3.2. Definitions of variables related to behaviors and condition.

Dominant male	Male of the tank pair with higher dominance score
Dominance score	Number of times (out of eight observations) the male exhibited more aggressive behaviors than his tankmate.
Spawning success	Number of spawning events male obtained/total number of spawning events in male's tank
Total male-male aggression	Male-male fin flares, sigmoids, chases and attacks summed across both males
Total male-female aggression	Male-female chases and attacks, summed across both males
Spawning events	Number of spawning events observed
Body condition	Residuals from the regression of $\log_{10}$(mass) on $\log_{10}$(standard length)
Infection status	Yes/no infection with one or more acanthocephalans
Parasite load	Total number of acanthocephalans

Table 3.3. Average counts (standard deviations) of male-male and male-female interactions for dominant and subdominant fish summed over the eight observations.

	dominant male	subdominant male
length (mm)	34.2 (4.1)	33.3 (4.7)
mass (g)	0.6035 (0.23)	0.5553 (0.23)
male-male flare	63.3 (72.7)	20.7 (20.7)
male-male chase	32.6 (31.1)	8.6 (17.2)
male-male attack	24.9 (22.4)	7.6 (15.5)
male-male sigmoid	2.6 (3.4)	0.7 (1.5)
circle fight	0.8 (2.1)	
male-female flare	24.2 (21.4)	8.8 (19.5)
male-female chase	11.6 (14.7)	3.3 (7.8)
male-female attack	8.8 (10.1)	3.6 (7.2)
courting	38.1 (32.8)	14.5 (31.2)
spawn	0.9 (1.2)	0.24 (2.1)
male-male-female chase	0.92 (2.3)	

Figure 3.1. Variation in anal fin pterins (a) anal fin melanin (b), and caudal fin carotenoids (c) in male *Lucania goodei.*

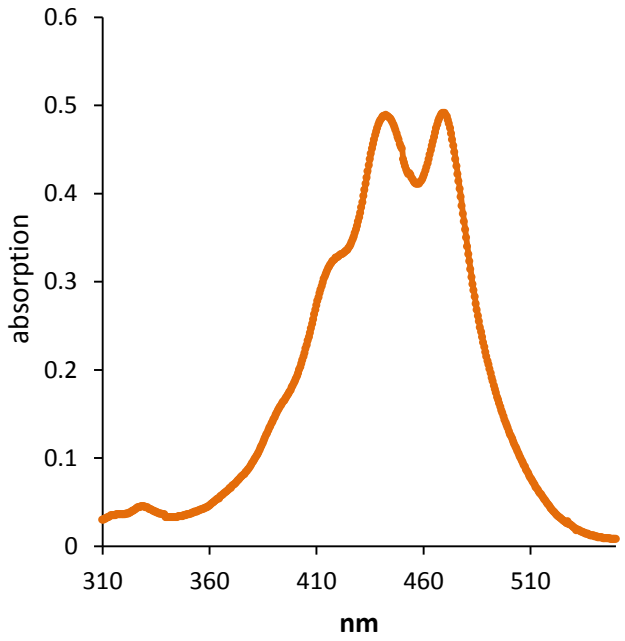


Figure 3.2. A representative caudal fin absorption spectra indicating carotenoid pigmentation.

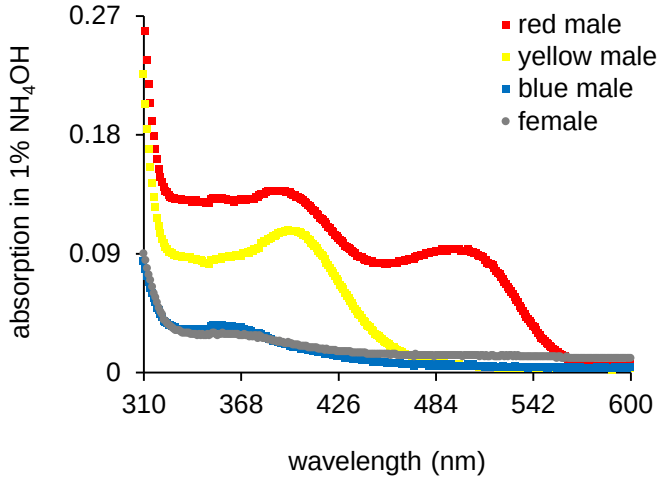


Figure 3.3. Representative absorption spectra of *Lucania goodei* anal fins indicate pterin pigment content in red and yellow morphs. Yellow pigment absorption peaks at 398 nm while red pigment peaks at 498 nm.

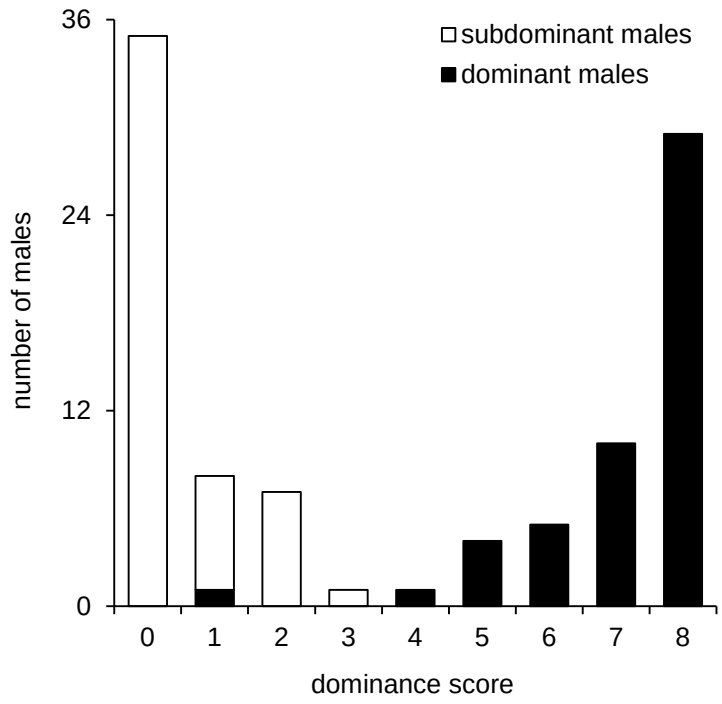


Figure 3.4. Number of times in eight observations that a male was scored as the dominant member of the male-male pair. When no activity from either male was recorded, neither male was scored as dominant. The male with the higher dominance score of the pair was identified as the dominant male.

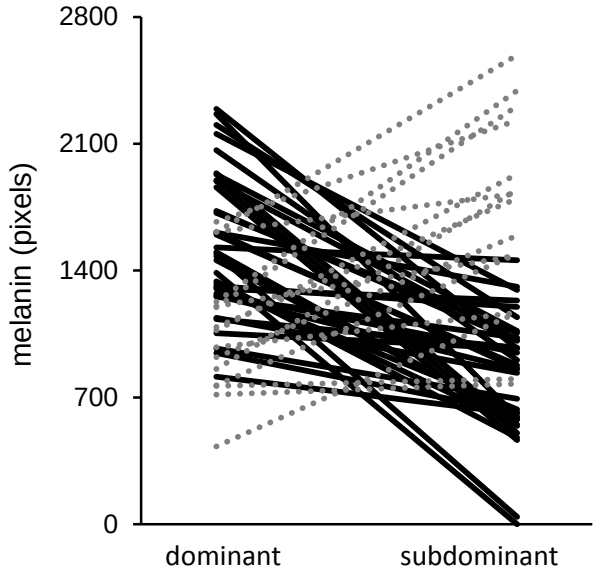


Figure 3.5. Dominant males have more melanin than their subdominant partners (connected by lines drawn) on the distal portion of their anal fins in photographs taken after behavioral observations.

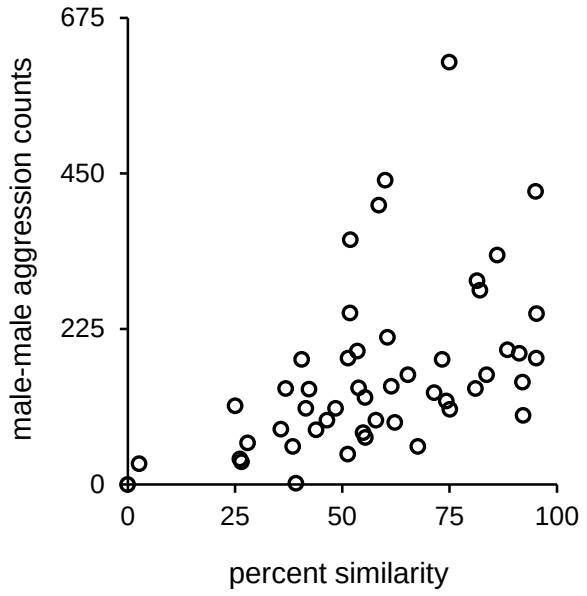


Figure 3.6. Percent similarity in anal fin melanin in photographs is correlated with the total number of aggressive interactions (male-male fin flares, sigmoids, chases, and attacks summed for both males).

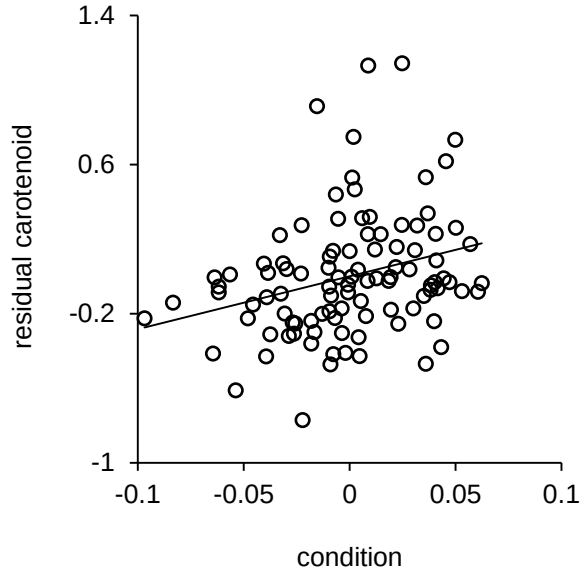
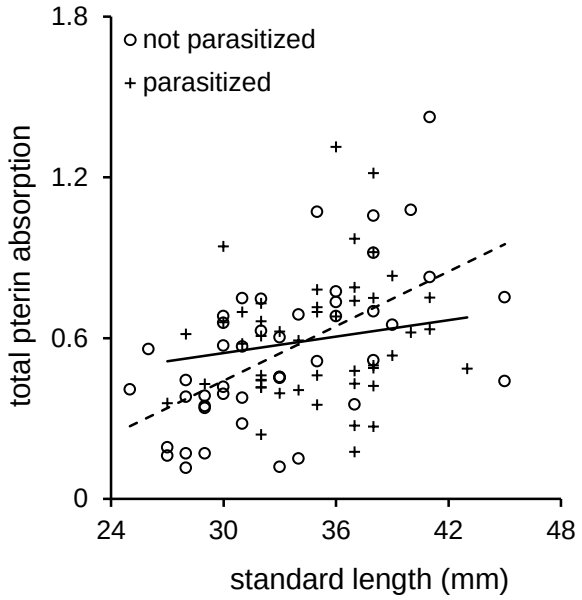


Figure 3.7. Male body condition is correlated with size-corrected caudal carotenoid pigment.

(a.)



(b.)

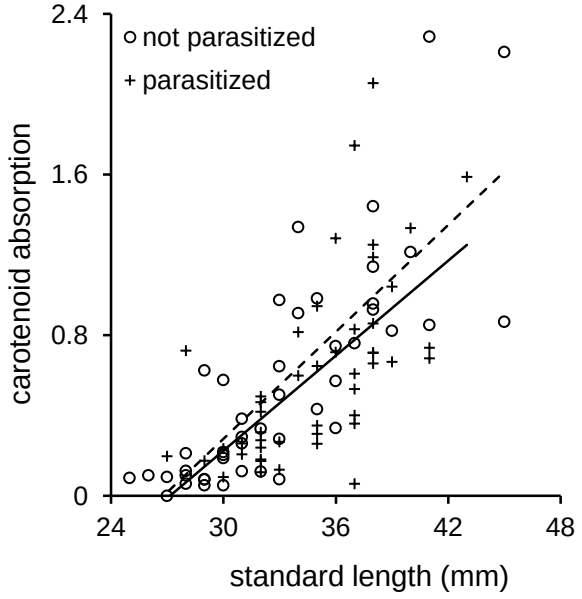


Figure 3.8. Interaction between total pterin (a) and carotenoid pigmentation (b) and length and their effects on acanthocephalan infection. Solid line is regression for infected individuals and dotted line is regression for uninfected individual.

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CHAPTER 4

VARIATIONS IN FIN PIGMENTATION HAVE FITNESS EFFECTS IN THE BLUEFIN KILLIFISH

ABSTRACT

Male bluefin killifish (*Lucania goodei*) vary in anal fin color morph (red/yellow) as well as in degree of pigmentation in the anal and caudal fins. Using a breeding experiment, we examined how the anal fin polymorphism is maintained and simultaneously assessed the fitness effects of degree of pigmentation (pterin, melanin, and carotenoid) in each male. We manipulated the ratios of red and yellow morphs in breeding populations housed in a greenhouse and determined if morph rarity conferred a fitness advantage by identifying paternity in the resulting fry. We also tested whether males with higher levels of melanin, pterin, and carotenoid pigments had increased fitness. We found no evidence of negative frequency-dependent selection on anal fin morph. However, red morph males did sire more offspring on sinking rather than floating spawning substrates when rare. This suggests lighting differences between the two microhabitats that affect conspicuousness may influence morph success. In addition to noting morph effects, we also found that males with more anal fin pigmentation (both pterin and melanin) and caudal fin pigmentation (carotenoid) sired more offspring, reinforcing the notion that pigmentation plays an important role in fitness.

INTRODUCTION

Individual variation is everywhere in nature. Whether that variation is found in highly differentiated categories or across a subtle cline, its existence still presents scientists with a paradox: how can this variation exist when selection and drift are constantly acting to remove variation within populations (Mitchell-Olds et al. 2007)? A number of mechanisms have been proposed to account for the existence of visually discrete variants (polymorphisms) within populations, but by far the best-supported of these has been negative frequency-dependent selection (Horth and Travis 2002; Roulin and Bize 2007; Fitzpatrick et al. 2007). In the case of negative frequency-dependence, selection in favor rare morphs prevents variation from being lost. This can come in the form of a mating advantage for rare morphs, as in guppies (Farr 1977; Hughes et al. 1999), or in the form of increased predation on common morphs (Olendorf et al. 2006) or increased competition from like morphs (Dijkstra et al. 2008).

In other cases, variation falls along a gradient rather than discrete classes, and condition-dependence has often been cited to explain this type of variation. Males with high trait values are preferred by females, but only males in good condition (high quality males) can produce high values of the trait (reviewed in Andersson 1994), such as with bird song quality (e.g. Forstmeier et al. 2002). Therefore, variation in condition amongst males correlates with variation in trait values (but see Grether et al. 2001). Of course, condition-dependence can also lead to distinct polymorphisms, as exemplified by horned beetles, where difference in nutrition quality can lead to polymorphism (or more aptly polyphenism) in horn size (Moczek 1998).

We chose to examine both polymorphic and continuous variations in fig pigmentation in the bluefin killifish (*Lucania goodei*). The bluefin killifish is a small, freshwater fish found throughout the southeast United States (Page and Burr 1991). Males compete for territories and access to females, who lay their eggs in small batches across multiple males'

territories (Fuller and Travis 2001). Males of this species have highly variable fins. The anal fin varies both in color class (red, yellow, or blue) and degree of pigmentation. The distal end of the anal fin has a melanic stripe that varies in size. In addition, the fish's caudal fin varies in amount of orange carotenoid pigmentation.

The function of the male anal fin color polymorphism has been the subject of interest in previous studies. These studies have demonstrated that morph prevalence is strongly tied to environmental conditions, where blue and red morphs are much more common in tannin-stained waters, and yellow morphs are more common in clear springs (Fuller 2002). Breeding studies have demonstrated that yellow is dominant to red, with one gene largely determining red/yellow phenotype and expression of blue coloration having both environmental and genetic components (Fuller and Travis 2004). Different morphs do not obviously have different behavioral types or mating strategies (McGhee and Travis 2010). Thus, it has been suggested that negative frequency-dependent sexual selection may maintain the polymorphism.

We examined the possibility of negative frequency-dependent selection in this species by manipulating the ratios of red and yellow males in a breeding experiment to determine if rare morph males sire more offspring. A previous study in *L. goodei* showed no evidence of negative frequency-dependent selection: red males sired more offspring when rare, but yellow males did not (Fuller and Johnson 2009). However, that experiment had short comings that were addressed in the current study. First, algal blooms occurred in some, but not all, of the experimental tanks, and this may have altered fish perception of male anal fin morph. Secondly, the paternity analysis only allowed assignment of offspring to the rare or common morphs in the tank as a class, rather than to specific fathers and mothers, which would have led to more detailed analysis of factors influencing levels of parentage.

In the current experiment, we manipulated male morph ratios to assess if rare morphs had more offspring than common morphs. Importantly, we were also able to examine how other color variation also affected paternity by measuring the amount of pigment (pterin, melanin, and carotenoid) in the anal and caudal fins of potential sires. We were also able to see how male health, as measured by overall condition and parasite load, affected paternity. This gave us a detailed portrait of how individual color variation in anal fin morph and degree of pigmentation influences fitness.

METHODS

Experimental setup

Our primary goal was to test for negative frequency dependence. To do this, we created two experimental treatments - one where red males were rare (1 red male, 5 yellow males, and 6 females) and another where yellow males were rare (1 yellow male, 5 red males, and 6 females). We performed 7 replicates of each treatment resulting in 14 experimental breeding populations. In addition, our experiment allowed us to test the degree to which pigments in male *Lucania goodei* influence mating success.

The fish used in this experiment were captured with a seine net in May of 2011 from the Upper Bridge population in the Walkula River, near Tallahassee, Florida. This population is polymorphic for color, with blue males being very rare and yellow males being more abundant than red males (Fuller 2002). The fish were transported back to the University of Illinois and housed briefly in a communal cattle tank (~300 L) before being moved to twelve experimental white cattle tanks (~300 L), which were housed in a glass greenhouse and exposed to natural lighting conditions. UV sterilizers attached to the tanks prevented algal blooms and maintained water clarity. The tanks contained six floating and

six sinking yarn mops, which the fish use for shelter and spawning substrate. Fish were fed commercial flake food, frozen daphnia, frozen adult *Artemia*, and blackworms. After six weeks, two replicates were concluded, and two new replicates were begun at that time bringing the total to fourteen. Thus, 10 replicates were run for 8 weeks, two were run for ~ 6 weeks, and two were run for ~2 weeks (Table 4.1).

The yarn mops in each tank were searched at least three times a week for eggs. Eggs were removed and maintained in a dilute solution of methylene blue until fry hatched out. Fry were fed baby *Artemia* for an additional three weeks after hatching. They were then stored in ethanol and frozen until DNA could be extracted using a standard protocol.

At the conclusion of the experiment, all adult fish were euthanized with an overdose of MS-222. Standard length and wet weight of each individual were recorded. Males were placed against a white background with a color standard, and a digital picture was taken of the left side of each male. The caudal and anal fin were removed and spread out on a glass slide. Rough measurements of each fin were taken by treating the fin as a parallelogram and noting length of its proximal and distal ends and the distance between the two. The fins were stored at -80 ˚C until pigment could be quantified. The caudal peduncles of all adults were removed and stored in ethanol at -80 ˚C until DNA was extracted. The adults were stored at -80 ˚C in ethanol until a later time, when they were dissected under a microscope. At that time, the number of Acanthocephalan parasites in the body cavity were noted.

Coloration analysis

Absorption spectroscopy was utilized to quantify carotenoid and pterin pigmentation in the fins. To measure caudal fin carotenoid pigment, the fin was ground in 350 ul of 1% NH_4OH in a test tube using a pestle. One ml of a 1:1 mixture of hexane:tert butyl methyl ether was then added to extract the pigment. The absorption of this layer was measured on a spectrophotometer, and the height of the absorption peak at 445 nm was used to quantify

carotenoid levels. To quantify the anal fin pigment, the anal fin was ground in 400 ul of 1% NH_4OH, and absorption was measured in a spectrophotometer. Yellow pterin pigment was quantified by absorption at 398 nm while red pterin pigment was quantified by absorption at 498 nm. Total anal fin pterin was measured as red and yellow pterin (absorption) summed. Yellow males express only the yellow pterin. Red males express both the yellow and red pterin (Chapter 3).

Anal fin melanin could not be analyzed using absorption spectroscopy, so digital picture analysis in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, imahej.nih.gov/ij/) was used instead. Small differences in magnification between pictures were corrected for by scaling each image to a size standard. The anal fin was isolated using the freehand selection tool, and the image was converted to black and white using the adjust threshold function and selecting black and white threshold color. The image was then converted to a binary image, and the area of the black band was calculated with the measurement tool.

Parentage analysis

Parents and offspring were typed at three highly polymorphic microsatellite loci: CA (Fuller and Johnson 2009), AC17 (Burg et al. 2002), and Lg1 (Creer and Trexler 2006). Forward primers were labeled with VIC (CA), 6FAM (AC17), or Pet (Lg1). The loci were amplified in one multiplex reaction according to the standard protocol in the QIAGEN Multiplex PCR Kit. The PCR products were run on an ABI Prism 3730xl Analyzer at the University of Illinois' W.M. Keck Center for Comparative and Functional Genomics. Fragment sizes were scored using Applied Biosystems GeneMapper and verified manually. We then used CERVUS V 3.0.3 (fieldgenetics.com) to assign parentage to the fry (Kalinowski et al. 2007). Each cattle tank was analyzed separately, and offspring were assigned parentage based on 80% likelihood. Only a small number of offspring (38 of 1051)

failed to have parentage assigned to them, due to either unresolvable parentage or poor DNA quality.

Many replicates experienced adult mortality. These individuals were included in the CERVUS parentage simulations as un-sampled potential parents, but in most cases, these individuals did not contribute any offspring, and we treated the replicate as having been formed without them. In three cases (three males and one female), the deceased individual did leave a notable number of offspring, and we were able to reconstruct his or her genotype, which helped further identify parentage. We were able to deduce the color morph of the missing males by examining the body and/or deducing it from the other morphs in the tank. However, we were unable to measure pigmentation, and our sample sizes reflect this. In other cases, individuals with pale fin coloration were initially misidentified as the wrong sex or morph. This altered our gender and morph ratios, but it did not affect our ability to detect the effect of pigmentation on paternity, and in fact more accurately represents the pigment variation found in nature.

Statistical analysis

We first report basic statistics on paternity, the skew in reproductive success in males and females, and general associations between size, condition, and mating success. We measured reproductive skew (S) separately for males and females in each replicate using Keller and Vargo's (1993) formula that results in a value from 0 (no skew) to 1:

$$S = \frac{v \cdot N_b + N_n}{N_b + N_n}$$

where N_b is the number of adults that bred at least one offspring, N_n is the number of individuals assigned zero offspring, and v is the variance among breeders in proportion of total offspring assigned parentage, divided by N_b (Table 1). We tested for differences in reproductive skew between males and females using a general linear model (proc glm). There were 28 data points in this analysis (each sex in each tank). We also tested for

differences in male reproductive skew between our treatments (red rare, yellow rare) across our 14 cattle tanks.

Our primary goal was to test for negative frequency dependence, which predicts that males will have their highest mating success when rare. To do this, we constructed a model that considered male color morph (red or yellow) and the tank treatment to which the individual belong (common tank color - i.e., yellow rare or red rare) and their interaction. Negative frequency dependence predicts a statistically significant interaction between these two effects (color morph and common tank color) where red males have their highest fitness in tanks where yellow males are common (and vice versa for yellow). We used a generalized linear model in SAS that assumed a binomial model where male paternity was the number of offspring assigned to him in relation to the total number of offspring genotyped from the tank. To control for the fact that that there are multiple males in a given tank, we treated tank as a repeated factor (i.e. males from a given tank were considered relative to one another). We also repeated the analysis where we considered the patterns in paternity for offspring that came from floating mops and bottom mops for rare and common males. In this case, we used modeled offspring sired in bottom mops in relation to total offspring sired for that male from both mops (and vice versa for offspring from top mops). We also modeled the difference from expected in the assigned proportion of offspring from the bottom mops for each male with one or more offspring.

Our second goal was to test whether there were any aspects of male phenotype (beyond their assigned color morph and rarity) that predicted male mating success. Here, we looked for correlations between mating success, male condition, anal fin size, and various measures of pigmentation including melanin, carotenoid, and pterin. The condition of each fish was calculated as the residuals of the $\log_{10}$ of weight regressed on the $\log_{10}$ of length (Bolger and Connoly 1989). Anal and caudal fin size in males was calculated by combining

the three measurements made of the fin into one principle component (using proc princomp) in which all three loaded positively. In order to test for correlations between pigments, Pearsons correlations were used with length as a partial correlate. To test for the effects of different pigment quantities on male paternity, a generalized linear model (proc genmod) was used. In most cases, a model was used that measured the variable of interest's effect on the number of fry assigned paternity to each male out of the total number of fry assigned parentage in his tank. This model used a binary distribution and the logit link function. We also tested variables' effects on the binary outcome of whether or not a male was detected having any offspring at all. When the result being tested was not a binary outcome (i.e. number of offspring rather than proportion), a normal distribution was assumed. For all of these models, tank was included as a repeated factor to account for correlations within tanks. Sample sizes included 14 tanks, containing a total of 87 males or 72 females. However, size and pigment values from three males are missing due to mortality (see above). Additionally, one carotenoid outlier (more than six standard deviations from the mean) was removed, and tests involving carotenoids do not include this individual unless otherwise noted.

RESULTS

Basic measures of reproduction

We identified parentage in a large number of fry (Table 1). In total, 1560 eggs were collected across the experiment (1340 from top mops and 220 from bottom mops). From those eggs, 1060 fry hatched and survived long enough to have DNA extracted. A subset of those (1051) were typed, and of those, 1011 (96%) were successfully assigned parentage by CERVUS at 80% confidence level or above.

Reproductive skew did not differ between males and females ($F_{1,26} = 1.37$, P = 0.25), nor did it vary between tanks in which red or yellow males were rare ($F_{1,12} = 3.1$, P = 0.10) (Table1). Neither length ($X^2_1 = 0.83$, P = 0.36) nor male mass ($X^2_1 = 1.28$, P= 0.25) affected the proportion of offspring assigned to each male or the numbers assigned to each male (length: $X^2_1 = 0.51$, P= 0.48; mass: $X^2_1 = 1.60$, P= 0.21). Fish condition had no impact on the proportion of fry assigned parentage in either males ($X^2_1 = 0.96$ P = 0.33) or females ($X^2_1 = 3.02$ P = 0.082). However, high condition females had significantly higher offspring numbers ($X^2_1 = 4.35$ P = 0.037) while males did not ($X^2_1 = 2.69$ P = 0.10).

Testing Negative Frequency Dependence

Red and yellow morph males sired equivalent proportions of offspring overall ($X^2_1 = 1.37$, P = 0.24). In addition, it appears that morph rarity did not give a male a mating advantage over common color morphs. In fact, males of the rarer color were actually assigned a smaller proportion of offspring, although this was not significant after using tank as a repeated factor ($X^2_1 = 2.02$, P = 0.16). Similarly, the interaction between male color and the majority color of the tank in proportion of tank offspring sired was not significant ($X^2_1 = 2.71$, P = 0.1).

Red color morphs that successfully sired at least one offspring did shift to siring a greater proportion of their offspring in bottom mops (vs. floating) when they were the rare morph in the tank ($X^2_1 = 3.64$, P = 0.0566) (Figure 4.1). Red males also sired a greater proportion of the offspring from the bottom mops than expected when they were rare ($X^2_1 = 5.4$, P = 0.02) (Figure 4.2). This can be interpreted as implying that when reds were rare, they sired a greater proportion of eggs on bottom mops than expected. However, when reds were common, both red and yellow morphs sired equivalent numbers.

There were no obvious differences between tanks where red or yellow morphs were in the majority. There was no difference in number of eggs laid in these tanks after correcting

for experimental duration and the number of females in the tanks ($F_{1,12}$ = 1.54 P = 0.24). There was also no difference in the proportion of eggs that were laid on the bottom mops between red and yellow majority tanks (X^2_1 = 0.9, P = 0.33).

Interestingly, red males had significantly larger anal fins than yellow males ($F_{1,82}$ = 12.53, P = 0.0007), but caudal fin size did not differ ($F_{1,82}$ = 0.0, P = 0.97). The fin size difference was not caused by overall size differences in the two morphs as red males were no longer (P=0.19) or heavier (P=0.44) or in better condition (P = 0.69) than yellow males. Large fin size did not significantly increase the proportion of offspring sired, however (X^2_1 = 1.97, P = 0.16).

Pigmentation

Males in each tank were assigned color morph (red/yellow by AJ). Visual assignment matched the absorption spectroscopy data from the anal fins: red morph males had significantly more red pterin than yellow males ($F_{1,82}$ = 63.9, P < 0.0001). Red and yellow males did not differ in amount of carotenoid (P = 1.0), black (P = 0.33) or yellow pterin (P = 0.69). Red males did have more total pterin ($F_{1,82}$ = 5.39, P = 0.023), suggesting that these males, because they produce the supplementary red pigment, are more pigmented overall in the anal fin.

None of the pigments were correlated with fish standard length, except for carotenoid, which was positively correlated with length after a single outlier was removed. Nonetheless, we used length as a partial correlate to correct for size and determine if the pigments were correlated with each other. Anal fin melanin was positively correlated with yellow pterin (N = 84, r = 0.27, P = 0.01), red pterin in red morphs (N = 41, r = 0.59, P < 0.0001), and total pterin (N = 84, r = 0.57, P < 0.0001). The correlation between melanin and carotenoid was marginal (N = 83, r = 0.18, P = 0.091). All the measures of pterins (yellow, red, and total pterin), were highly positively correlated with each other (P < 0.0001). Carotenoid levels

were positively correlated with yellow pterin (N = 83, r = 0.27, P = 0.015) and total pterin (N = 83, r = 0.25, P = 0.022), but not red pterin amongst red males (N = 40, r = 0.14 P = 0.38).

We tested whether each pigment was correlated with condition using pigment residuals on length (Figure 4.3). Male condition was positively correlated with melanin (N = 84, r = 0.34 P = 0.002), yellow pterin (N = 84, r = 0.36 P = 0.0004) and total pterin (N = 84, r = 0.36, P = 0.0007), but not red pterin amongst red individuals (N = 41, r = 0.20 P = 0.19) or carotenoid (N = 83, r = 0.14, P = 0.19).

We examined the effect of each pigment on the proportion of offspring sired. Males with higher amounts of melanin sired a higher proportion of the offspring (X^2_1 = 4.53 P = 0.033). Males with more yellow pterin (X^2_1 = 5.16 P = 0.023) and total pterin (X^2_1 =4.86 P = 0.027) also sired a higher proportion. More red pterin did not result in a higher proportion of offspring, (X^2_1 = 2.37 P = 0.12), even after limiting the analysis to just red males (X^2_1 = 0.88 P = 0.34). Higher carotenoid levels also resulted in a higher proportion of sired offspring (X^2_1= 3.94 P = 0.047). Values remained significant after using residuals corrected for length (melanin: X^2_1 = 4.57 P = 0.033; yellow pterin: X^2_1 = 4.53, P = 0.033; total pterin: X^2_1 = 4.54, P = 0.033), except for carotenoid, which became marginally non-significant (X^2_1 = 3.45 P = 0.063) (Figure 4.4).

We performed a similar binary analysis on whether or not the male produced any offspring at all (Figure 4.4). In this case, all the pigment values were once again highly significant (melain: X^2_1 = 4.27, P = 0.038; yellow pterin: X^2_1 = 4.31 P = 0.038; total pterin: X^2_1 = 3.84 P = 0.050, red pterin amongst reds: X^2_1 = 4.68 P = 0.031; carotenoid X^2_1 = 5.57, P = 0.018) even after using residuals correcting for length (melanin: X^2_1 = 4.25 P 0.040; yellow pterin: X^2_1 = 4.19, P = 0.041; total pterin: X^2_1 = 0.76, P = 0.053; red pterin amongst reds: X^2_1 = 4.72, P = 0.030; carotenoid: X^2_1 = 4.55, P = 0.033).

DISCUSSION

By manipulating the ratios of red and yellow morphs in *Lucania goodei*, we were able to detect whether rare morph males have a mating advantage that results in increased paternity. We show here, in corroboration with previous results (Fuller and Johnson 2009), that rare males have no significant mating advantage. While our results could have been affected by mortality in eggs or fry that hindered all offspring from having parentage assigned, only positive frequency-dependent selection on offspring mortality could have altered our experimental results, and this is extremely unlikely. We can therefore be reasonably certain that negative frequency-dependent sexual selection is not operating to maintain the red/yellow anal fin polymorphism in *Lucania goodei*.

The results in *L. goodei* stand in contrast to the guppy, *P. reticulata*, where negative frequency-dependent selection occurs through at least two known mechanisms (mating and predation) (Hughes et al. 1999; Olendorf et al. 2004). The source of this disparity might the different effective population sizes of the two species. Guppy populations are small and can become quite isolated, especially during the dry season (Griffiths and Magurran 1997). In addition, numerous studies have demonstrated that guppies can suffer from inbreeding depression (van Oosterhout et al. 2003; Mariette et al. 2006; Pitcher et al. 2008; Johnson et al. 2010). Therefore, it makes sense for behaviors that facilitate inbreeding avoidance, such as a preference for rare males, to evolve. In contrast, bluefin killifish have extremely large population sizes (Turner et al. 1999), and females actively allocate their eggs across multiple males (Fuller 2001), which lessons the potential consequences of inbreeding. Without the potential for inbreeding depression, female bluefin killifish might not strongly benefit from mate discrimination based on morph abundance.

If negative frequency-dependent sexual selection is not operating to maintain this red/yellow anal fin polymorphism in *L. goodei*, how has it persisted? We did observe that when rare, red males sired more offspring on the bottom mops (Figure 4.1,4.2). It may be that microhabitat variation in light quality facilitates the co-existence of these morphs. Our previous research has demonstrated that *L. goodei* have a visual system that is attracted to colors that contrast with available light. For example, in tannin-stained waters, which quickly attenuate short wavelengths, the species is more attracted to blue, while in clear waters they are more attracted to red (Fuller et al. 2010, Chapter 5). In clear spring water, (like that of our source population and which our cattle tanks mimicked), longer wavelengths are attenuated more quickly than shorter wavelengths. Thus, there are relatively fewer red wavelengths in spawning substrates at the bottom of the water column than at the top. Our measurements here indicate that red males have larger anal fins. They may find greater mating success by attracting more attention with their larger, more contrasting anal fins at lower depths. This advantage was probably more prevalent in our tanks where red males were rare because females were better able to contrast them with yellow males, or they may have been more readily excluded by yellow males from top mops. (Though the fish do not appear to greatly prefer one spawning substrate over another, in captivity top mops are preferable spawning substrates to bottom mops because of increased conspecific cannibalism on bottom mops (Sandkam and Fuller 2011). Of course, this would indicate that red males should be found more often at depth, which is not readily apparent in the wild (Fuller 2001). Nonetheless, this suggests a potentially fruitful avenue of future research.

Effects of pigmentation

We noticed a strong effect of pigmentation, irrespective of size, on male paternity. More pigmented males were more likely to have at least one offspring, and they produced more offspring in general (Figure 4.4). Our previous work looked at the effect of

pigmentation on dominance and found a strong effect of degree of anal fin melanism on dominance, and thus access to females (Chapter 3). The results of this experiment confirm those results and indicate that dominance in this species can directly translate into increased offspring numbers, even when females can presumably avoid aggressive males by hiding or preferentially mating with other males.

We also detected increased paternity in males with high levels of carotenoids and pterins. Our previous research indicated that while these pigments were unrelated to dominance, higher levels still led to increased spawning events with females (Chapter 3). Hence, this experiment provides further support for the hypothesis that these pigments are important for male mating success and may be particularly important for attracting females. However, exactly what these pigments are signaling is unclear. We argued previously that higher levels of these pigments may be attractive to females because these pigments were signals of condition in the case of carotenoid, and parasite load in the case of both pterin and carotenoid abundance.

While we see evidence for female attraction to increased levels of pigments in our parentage analysis, we did not see evidence for the previously ascribed signaling functions in this data set. Condition was correlated with melanin and pterin in this data set, not carotenoid (Figure 4.3). We note that there was a positive relationship between carotenoid and condition, but that the result was not statistically significant ($r = 0.14$, $P = 0.19$). In our previous data set, carotenoid was highly positively correlated with condition and the relationship was statistically significant. Neither melanin nor pterin were positively associated with condition although pterin was associated with parasite load in our previous experiment. In this experiment, there was no relationship between acanthocephalan parasitism and either pterin or carotenoid levels.

We believe these contradictory results can be explained by the differences in the fish used for these experiments. While both used fish collected from the same population, the fish in this experiment had been in captivity for much shorter time, were in much poorer condition, and weighed on average 27% less. The fish in this experiment were much more heavily parasitized as well (82 % infected with acanthocephalans versus 51%). It may be that the fish were in such poor condition that measures previously insensitive to condition (melanin and pterin), were affected by fish health, and highly sensitive measures (carotenoid) became influenced by other factors that we did not measure, such as additional parasite species. It should also be noted that the relationship between parasite levels and pigmentation was driven by the largest fish in our previous work. As this experiment used smaller fish, it is not surprising that we did not get the same results. Our results show that the value of pigmentation as a signal of health and condition may vary due to natural conditions.

In sum, our results suggest strong selection for increased pigmentation of all types. What, then keeps all males from evolving high-level pigmentation? We have argued previously that melanin acts as a badge of status in territorial interactions with other males. In this case, repeated interactions with other males may be what keep it honest (Searcy and Nowicki 2005). The pigments also seem to be tied to varying degrees to condition, with higher condition males having more pigment.

Table 4.1. Desciptive statistics for each replicate tank.

red/yellow males	females	days run	eggs	proportion eggs bottom	fry analysed	offspring with parentage	male skew	female skew
1 / 4	7	15	40	0.18	31	31	0.090	0.222
1 / 5	6	41	110	0.14	62	61	0.381	0.044
1 / 5	4	43	114	0.20	64	63	0.019	0.076
1 / 5	6	37	115	0.16	68	63	0.088	0.199
1 / 5	4	56	99	0.17	66	65	0.130	0.560
1 / 5	6	55	97	0.22	81	80	0.147	0.060
2 / 6	4	43	123	0.03	70	65	0.212	0.084
4 / 1	6	57	60	0.10	45	44	0.556	0.221
4 / 3	5	56	133	0.14	115	115	0.033	0.040
5 / 1	5	43	109	0.15	76	72	0.403	0.293
5 / 1	6	19	112	0.33	88	86	0.119	0.107
5 / 1	5	41	188	0.09	101	98	0.530	0.220
6 / 2	4	55	125	0.06	72	71	0.352	0.064
6 / 1	3	56	135	0.10	112	99	0.172	0.035

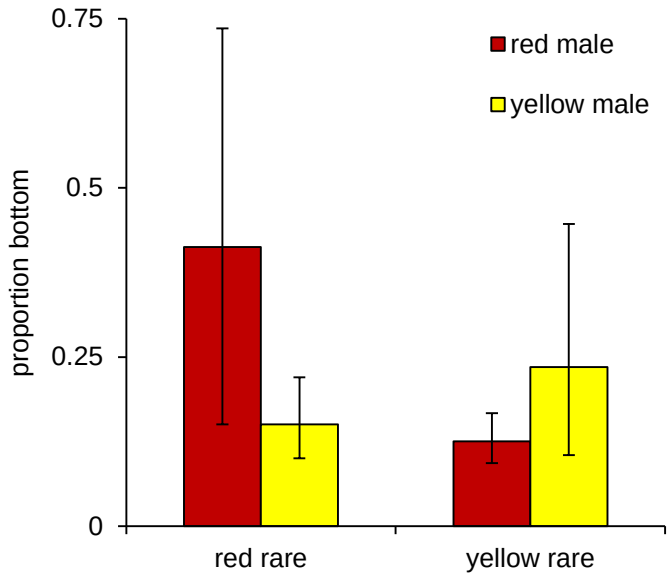

Figure 4.1. The average proportion (± 95% confidence interval) of a male's total offspring that were spawned on bottom mops as a function of male color and rarity of that color.

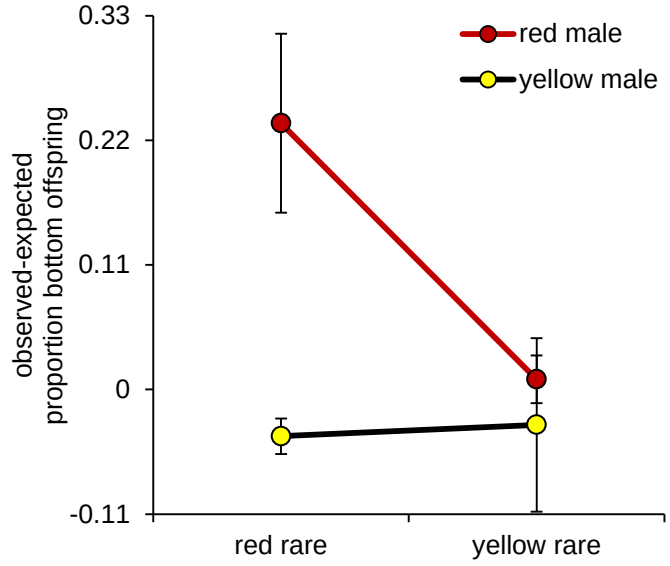

Figure 4.2. Average difference (± 95% confidence interval) between observed and expected proportion of offspring sired on bottom mops by male color and morph abundance.

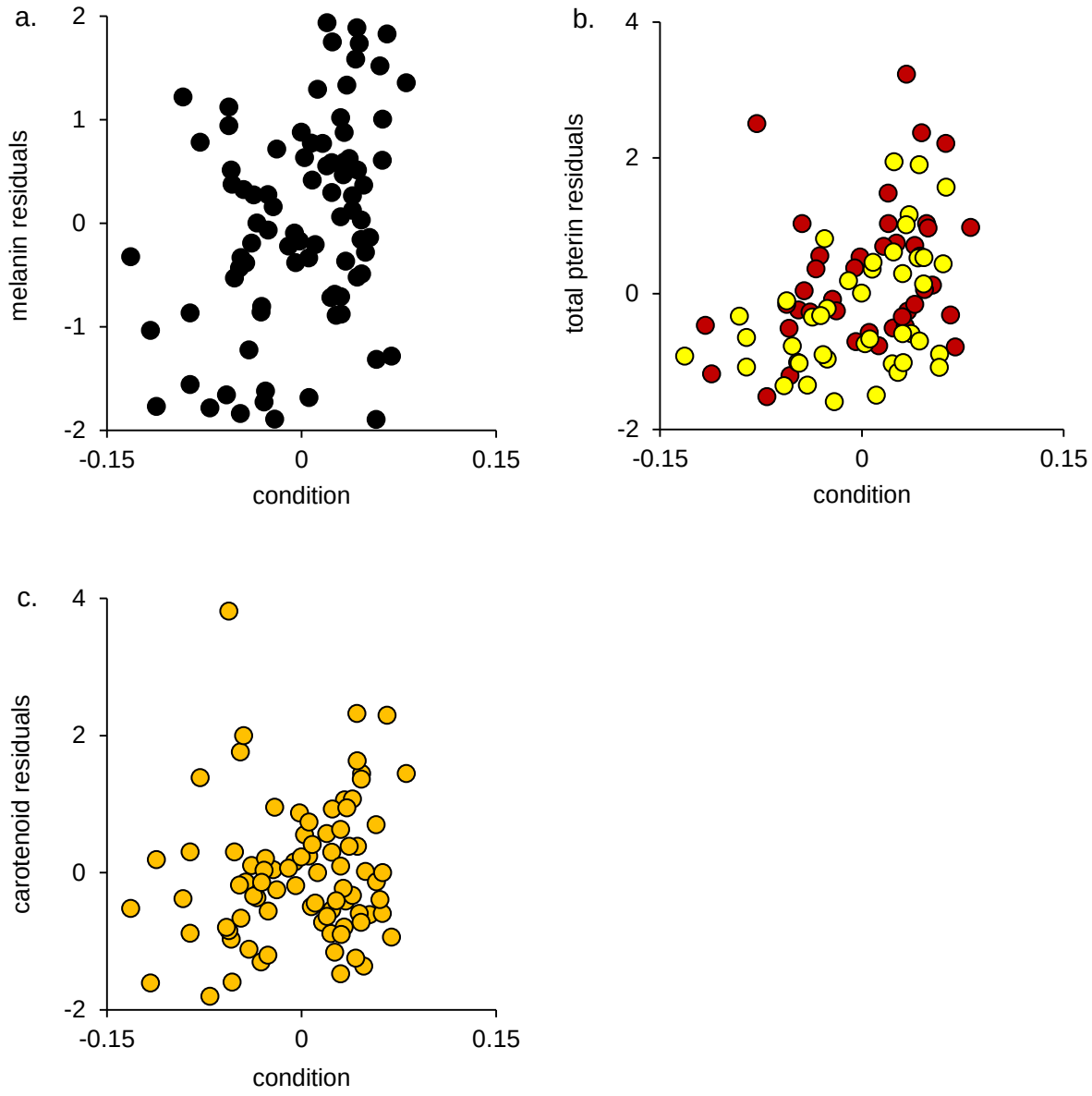

Figure 4.3. Relationship between condition and (a) melanin, (b) total pterin, and (c) carotenoid pigmentation. Pigment values are studentized residuals corrected for length. Symbol color in (b) refers to anal fin morph

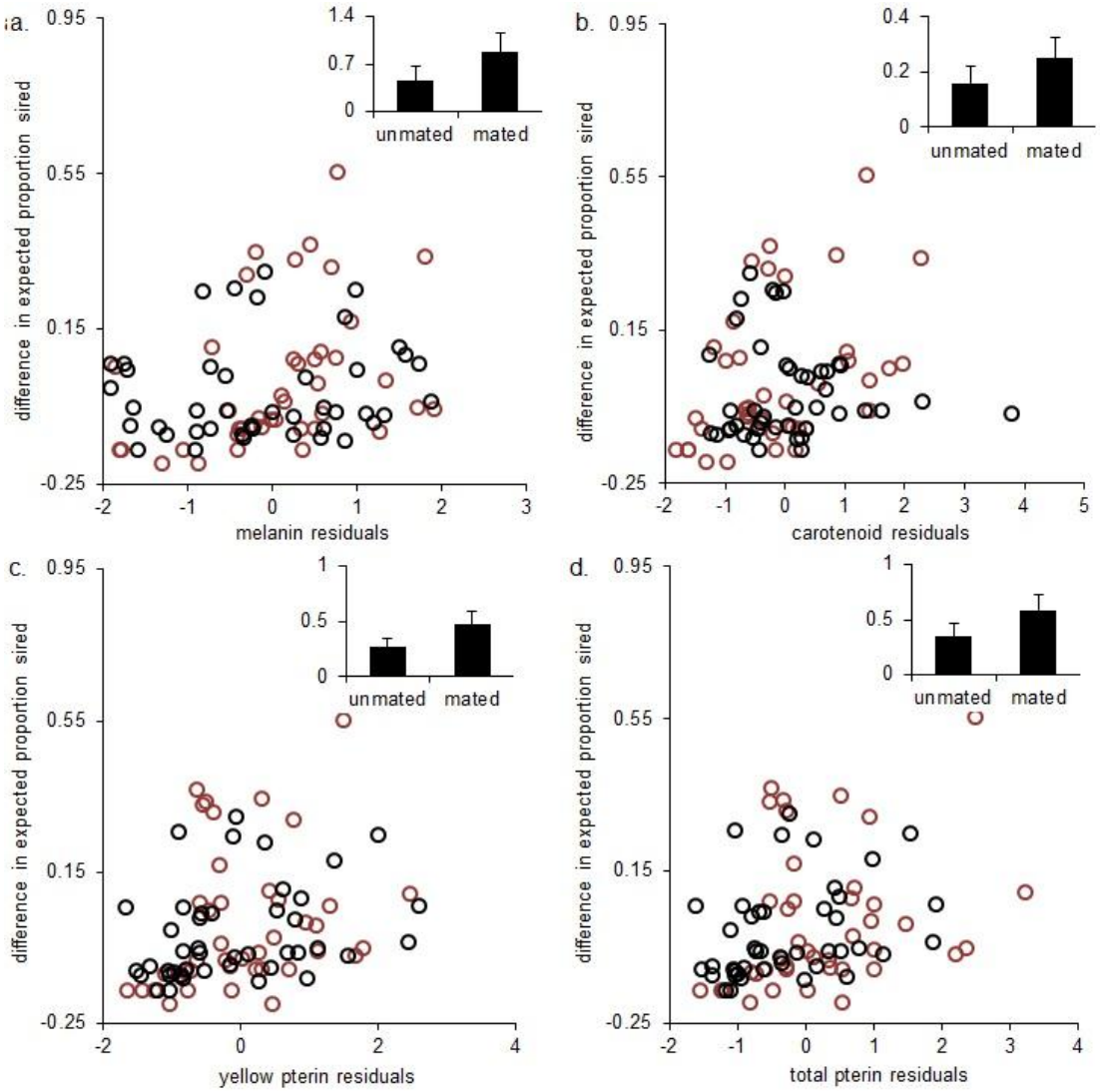

Figure 4.4. The effect of degree of pigmentation on difference between observed and expected proportion of offspring sired for (a) melanin, (b) carotenoid, (c) yellow pterin, and (d) total pterin. Black circles are values from yellow males while red circles are values from red males. Pigment levels are studentized residuals corrected for length. The bar graphs within are the uncorrected pigment level means of tank means of mated and unmated individuals (± standard deviation).

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CHAPTER 5

DIURNAL LIGHTING PATTERNS AND HABITAT ALTER OPSIN EXPRESSION AND COLOR PREFERENCES IN A KILLIFISH

ABSTRACT

We measured the diurnal pattern of cone opsin gene expression in the bluefin killifish (*Lucania goodei*) to see if overall or proportional opsin expression is tuned to match the daily blue-shift in light at dawn and dusk. LWS, RH2-1, RH2-2, and SWS2B (but not SWS1 or SWS2A) opsin expression was lowest at midnight and dawn and highest at midday and dusk, and the observed temporal shifts were many times larger than an accompanying difference in production of opsins in tannin-stained versus clear habitats. We also measured color preference in a foraging assay at dawn, midday, and dusk to determine if opsin expression influenced fish behavior. Rather than correlating with opsin expression, foraging behavior matched lighting conditions, with higher preferences for blue at noon and red at dawn/dusk, when these wavelengths are comparatively scarce and the contrast of these colors is increased. Our results suggest that *L. goodei* exhibit strong diurnal cycles of opsin gene expression, but that these are not correlated with light intensity or light color per say. Temporally variable preferences for different colors are probably the result of lighting environment rather than opsin production. These results may have implications for the color pattern diversity observed in these fish.

INTRODUCTION

Our visual world is extremely dynamic. From high noon to the depth of night, the amount of light available for vision can vary by a factor of over a billion (Davies et al. 2012). The ability to see food, mates, and predators across this vast range of conditions requires an incredibly plastic visual system that can respond to abrupt changes in light. The visual system that has evolved in vertebrates to deal with this complex process utilizes specialized photoreceptor cells—rods and cones. Rods are specialized for low-light vision while cones are specialized for light or color vision. The outer segments of these cells are composed of stacks of discs filled with a protein (opsin) linked to a light absorbing chromophore (retinol or 3-dehydroretinal). There are multiple classes of cone opsins, each of which has a wavelength range to which it is most sensitive (λ_{max}). By comparing the quantum catch of the different cone classes, the brain is able to deduce color (Bowmaker 2008).

Species evolve visual systems suitable for the lighting environment of their respective habitats to facilitate activities such as foraging and mate choice (Cummings and Partridge 2001; Horth 2007; Temple et al. 2010). This can happen through altering the number of different photoreceptor types found in the retina, or through mutations in the amino acid residues of an opsin that change the opsin's λ_{max} (Kelber et al. 2003; Osorio and Vorobyev 2005; Frentiu et al. 2007; Carleton 2009). But even over the course of an individual's lifetime, the visual system can be altered to match a changing environment. Simply switching the form of the chromophore can shift the λ_{max} of the photoreceptor by several nanometers, a process seen in many anadromous and catadromous fish (Beatty 1984). Many animals use various types of filters to affect the wavelengths of light that reach their photoreceptors. Some birds, reptiles, and lungfish have oil droplets in their cones that are filled with colored carotenoids. The oil absorbs shorter wavelengths and transmits only longer wavelengths (Bowmaker 2008). Positively correlating the density of carotenoids in the oil droplets with light levels can increase sensitivity in dim environments and color

discrimination in bright environments (Hart et al. 2006). Opsin expression can also change to match the lighting environment. For example, salmon switch the opsins their cones express as they move from shallow to deeper waters (Cheng and Flamarique 2004), and the black bream (*Acanthopagrus butcheri*) shifts opsin expression as it makes habitat changes from clear to deeper tannin-stained water (Shand et al. 2002; Shand et al. 2008).

Clearly, many organisms have the ability to alter opsin expression as they transition between different habitats (i.e. lighting environments). However, organisms also experience huge (yet predictable) fluctuations in lighting environment over the course of each day. As the sun changes position in the sky, both the absolute irradiance and the relative amounts of each wavelength of light change. During the day, higher wavelengths of visual light are represented in roughly equal proportions, with a gradual drop-off as wavelength values fall under 450 nm. At dawn and dusk, however, the available light becomes blue-shifted as the amount of orange light drops much faster than the amount of blue light (Munz and McFarland 1973; Endler 1991; Johnsen et al. 2006).

Organisms can alter their behavior to manage the effects of this temporal variation in visual environment. For example, guppies display closer to females in dim light (Long and Rosenqvist 1998) and preferentially court/mate early and late in the day, when they are most conspicuous to conspecifics but less likely to be seen by predators (Endler 1987). And some birds choose to perform courting displays under the lighting conditions that optimize contrast between their plumage and background (Endler and Thery 1996). These studies assume a constant visual system and behavioral modifications to ambient light conditions. Yet, the visual system itself might also vary with these changes in lighting environment and in turn also affect these behaviors. Cone cells shed part of their photopigment-containing outer segments every night and subsequently rebuild them during the day (Eckmiller 1997). The

extent to which this cycle correlates with light levels and alters visually-based behaviors is unclear.

We sought to determine if organisms deal with diel visual variation by manipulating the overall or relative amount of opsin they express in correspondence with the wavelengths of light available. In order to address this question, we utilized a fish that exhibits dynamic opsin expression to match its visual environment. The bluefin killifish, *Lucania goodei*, is an abundant diurnal freshwater fish living throughout the southeastern Unites States. This species is found across a wide variety of habitats that vary in lighting conditions. Populations in habitats with more dissolved hummic materials (tea-stained), which inhibit the transmission of short wavelengths of light, have fewer short-wavelength cones (Fuller et al. 2003) and express lower relative amounts of short-wavelength opsins (Fuller et al. 2004). Raising fry in different lighting conditions (tea-stained versus clear water) can induce a developmental change in the proportional amount of short-wavelength sensitive opsin expression (Fuller et al. 2005), as can simply switching fish from one water condition to another (Fuller and Claricoates 2011). This change in opsin expression is extremely rapid, happening in less than three days.

It is clear these killifish can respond over the course of a just a few days to different lighting conditions, but whether the fish adjust their opsins over shorter time periods to match the daily fluctuations inherent to their lighting environment is unknown. In this study, we addressed the following four questions. First, are there diurnal patterns in opsin expression that follow the daily patterns in light abundance where light (and opsin expression) are lowest at midnight, highest at midday, and intermediate at dawn and dusk? Second, we asked whether the proportional expression (i.e. the amount of each opsin relative to the total pool of opsins) varied over time or whether all of the opsins varied in a similar manner over the course of the day. Changes in proportional opsin expression are theoretically consistent with

changes in color sensitivity (Endler 1992). Third, we asked whether diurnal opsin expression varied among lighting habitats (i.e. clear vs. tea-stained water) and whether diurnal fluctuations or habitat changes had larger effects on opsin expression. Finally, we asked whether visually based behavior (attraction to different colored dots) varied as a function of time, lighting habitat, or their interaction and whether there was a strong relationship between shifts in opsin expression and behavior.

METHODS

The killifish, *Lucania goodei*, used for this experiment were collected in early October 2011 using dip nets and seines from Rum Island Park, Florida. This sight occurs on the Santa Fe River where two springs (Rum Island Springs and Blue Springs) join together. The site is known for being highly variable both spatially and temporally in the amount of dissolved hummic material. Thus, the source population is exposed to widely different visual environments, from clear to darkly tea-stained water. The fish were transported back to the University of Illinois and housed at 14 fish/tank in six separate 114 L tanks. Lipton instant decaffeinated tea was added to three of the tanks to match the visual environment to a tea-stained habitat while the other three tanks remained clear. The fish were housed in a greenhouse with siding that transmitted wavelengths from 385 nm to 800+ nm, ensuring that they were exposed to natural light cycles. At that time of year, sunrise and sunset are approximately 10.5 hours apart with some light visible (daylight plus nautical twilight) for approximately 12.5 hours/day. The fish lighting environment was supplemented with broad spectrum lamps on a 12 h light:dark cycle. Representative irradiance spectra of light conditions at noon and dawn/dusk are available in the supplemental (Supplemental Figure 5.1). Fish were allowed to acclimate to their tanks for 10 days before behavioral trials began.

To determine how time of day affected color preference behavior in the fish, the fish were given a color choice test. A plastic overhead sheet was painted with different colors of acrylic paint: red, orange, yellow, green, blue, black, and white. Fuller et al. (Fuller et al. 2010) describes the reflectance spectra of each color, except orange, which is included in the supplemental materials (Supplemental Figure 5.2). A standard hole-punch (6 mm) was used to punch out colored circles, and the circles were glued to a petri dish in a random order. The dish was placed in the center of the tank, and the number of times the fish pecked at each color was recorded for two minutes after the first peck. If no fish pecked at the dish for any time during the first 10 minutes of being exposed to the petri dish, the trial was discarded. Trials were conducted at three separate times: within an hour of dawn, midday, and dusk. We did not measure pecking behavior at midnight because the fish are not active at this time, and we would have had to disrupt the natural light cycle in order to measure the behavior. Each tank was measured at least once at all three times; however, the fish in one tank failed to peck at any circles during repeated trials for two of the time points. For each tank, no more than one measurement was taken per day, with at least 24 hours between each tank's successive measurements. Trials occurred over 10 days.

Following the behavioral trials, one fish from each of the six tanks was harvested for three successive days at four separate time points: within an hour of dawn, midday, dusk, and midnight (72 fish total). For the midnight collection, a red-light headlamp was used as a visual aid. Each fish was euthanized with an overdose of MS-222. Both eyes were removed, punctured, and stored in RNAlater until RNA could be extracted.

RNA was extracted from the eyes with trizol using the protocol described in (Fuller and Travis 2004; Fuller and Noa 2010; Fuller and Claricoates 2011) and stored at -80°C until we performed cDNA synthesis utilizing Superscript III. The transcription of six separate opsins was measured using specifically designed Taqman primers and probes. Elongation

factor 1-α (hereafter EF1-α) was also included as a housekeeping control (see (Fuller and Claricoates 2011) for sequences and accession numbers). Five distinct cone classes have been identified in *L. goodei*: UV (λ_{max}=359 nm), violet (λ_{max}=405 nm), blue (λ_{max}=455 nm), yellow (λ_{max}=539 nm), and red (λ_{max}=573 nm) (Fuller et al. 2003). Yokoyama and colleagues (Yokoyama et al. 2007) cloned the short-wavelength sensitive opsins and expressed them in vitro to determine their λ_{max} when combined with 11-cis retinol (SWS1 λ_{max}=354 nm, SWS2B λ_{max}= 397 nm, SWS2A λ_{max}=448 nm). Because these in-vitro λ_{max} values are similar to those observed by microspectrophotometry, Fuller and colleagues assumed that the five cone classes observed in *L. goodei* were SWS1 (UV), SWS2B (violet), SWS2A (blue), RH2-1 (yellow), and LWS (red). However, recently Fuller and Claricoates (Fuller and Claricoates 2011) discovered an RH2-2 sequence also existed, and its expression was much higher than expression measures of SWS2A. Thus, the blue cones observed in *L. goodei* are probably RH2-2 rather than SWS2A, while SWS2A is believed to be co-expressed at low levels with SWS2B in the violet cones (Fuller and Claricoates 2011).

For each of the six opsins (LWS, RH2-1, RH2-2, SWS2A, SWS2B, and SWS1) and EF1-α, three technical replicates were measured on an ABI Prism 7700 Sequence Detection System. The average critical threshold for each individual, after removing outliers, was utilized to estimate both proportional opsin gene expression (i.e. each opsin proportional to the total pool of all the fish's opsins) and relative opsin expression (i.e. each opsin relative to that fish's EF1-α expression). One out of 72 individuals did not run and was removed from further analysis.

To calculate the proportion of the total quantity of opsins that each individual opsin contributed, the following formula was used:

$$\frac{T_i}{T_{all}} = \frac{\frac{1}{(1+E_i)^{Ct_i}}}{\sum \frac{1}{(1+E_i)^{Ct_i}}}$$

In this case $\frac{T_i}{T_{all}}$ is the amount of each individual opsin for each fish divided by the sum of all opsins. The amount of each opsin was calculated using E_i as the efficiency of each opsin and Ct_i as the critical threshold obtained for each individual gene. Efficiencies are from Fuller and Claricoates (Fuller and Claricoates 2011), who utilized the same primers and probes and detection system and listed their efficiencies as one plus their calculated values.

The amount of each opsin relative to housekeeping gene EF1-α was also calculated using the following equation:

$$\frac{T_i}{T_{ef}} = \frac{\frac{1}{(1+E_i)^{Ct_i}}}{\frac{1}{(1+E_{ef})^{Ct_{ef}}}}$$

In this case $\frac{T_i}{T_{ef}}$ is the amount of the individual opsin for that fish relative to the amount of EF1-α, and the amount of each opsin is independent of the amount of the other opsins. The opsin amounts relative to EF1-α were log transformed to normalize the data.

All data were analysed using SAS v 9.3 (SAS Institute, Cary, NC, USA). Proc Mixed was used to analyse the opsin data. Time of day, water, and sex were treated as fixed effects. The day on which each individual was euthanized and the qRT-PCR plate on which each individual's cDNA was run were treated as random effects for all opsins. Sex had no effect on any opsin and was removed from the models. Similarly, no interactions were significant, and all were removed.

To analyse the behavioral data, all trials for each time of day were summed so that each tank had three separate values, one for each time. The number of pecks each color received out of the total number of pecks was modeled with Proc Genmod using water and time as fixed effects (Supplemental Table 5.1). The model used a binomial distribution and the logit link function and was corrected for overdispersion. Because of low numbers, models using the proportion of yellow, black, and white pecks failed to converge. To

determine if there was an overall preference for pecking at a certain time, the number of pecks for each tank at each time were averaged and then square root transformed to normalize the data. An ANOVA in Proc Mixed with tank as a random factor was used to see if there was an overall preference for time.

RESULTS

Opsin data

Opsin gene expression varied dramatically over the course of the day. There was no effect of time of day or water color on our control gene EF1-α ($P = 0.85$ and $P = 0.30$, respectively), and as a result, we were able to use it to standardize the amount of each opsin and the total amount of opsin expression summed. Total opsin levels were not strongly affected by water ($F_{1,57} = 3.43$, $P = 0.069$) but they were affected by time ($F_{3,57} = 21.06$ $P < 0.0001$) with midday and dusk having significantly higher expression values than midnight and dawn ($F_{1,57} = 62.74$, $P < 0.0001$). Most of the opsins followed this overall expression pattern: expression was low from midnight to dawn and high from noon to dusk (Figure 5.1a-f). LWS had the largest increase, with relative expression more than 2 times higher at midday/dusk than at midnight/dawn (Figure 5.1a). Expression of RH2-1, RH2-2, and SWS2B showed similar patterns. Intriguingly, SWS1 appeared to increase between dawn and midday in the clear water treatment (where shorter wavelengths are available), but did not increase in the tea-stained treatment. However, this result needs to be interpreted with caution as the interaction between water and time was not statistically significant ($F_{3,54} = 0.59$). SWS1 and SWS2A showed no significant effect of time (Table 5.1, Figure 5.1d, 5.1f).

Proportional opsin expression also varied with time, with the most striking differences occurring on the opsins at the extreme ends of the visible light spectrum (Table 5.1). The

proportional LWS and SWS1 expression mirrored each other. LWS proportional expression climbed after dawn, stabilized from midday to dusk, and fell back to starting levels overnight (Figure 5.1g). Proportional SWS1, however, fell during the morning hours, stabilized from midday to dusk, and began to climb once again after dark (Figure 5.1l). RH2-2, SWS2A, and SWS2B showed a similar pattern to SWS1, while there was no effect of time on RH2-1 proportional expression (Figure 5.1h-k). Therefore, the changes observed in proportional expression were caused by a strong increase in LWS expression from midday to dusk in concert with flat SWS1 expression over time.

Water color significantly affected opsin expression. Proportional expression of SWS1 was significantly lower in the tea-stained versus clear water, although the expression relative to EF1-α did not vary (Table 5.1, Figure 5.1f, 1l). The effect of water color on proportional SWS1 expression appears to be driven by the fact that LWS, RH2-1, and RH2-2 all had significantly more expression in tea-stained water by both proportional and relative measures, and SWS2A and SWS2B showed significantly more expression in tea-stained water in the data relative to EF1-α (Table 5.1, Figure 5.1).

Behavioral data

Fish pecked at the colored circles three times more frequently in tea-stained water. Of the 271 pecks observed, 50% were red. The average numbers of pecks per each observation (mean ± SE) were red (4.0 ± 1.5), orange (2.0 ± 0.4) blue (1.5 ± 0.5), green (0.5 ± 0.2), yellow (0.2 ± 0.1), white (0.09 ± 0.06), and black (0.03 ± 0.03). There was no significant difference in the amount of pecking observed at different times of day ($F_{2,8} = 1.78$, $P = 0.23$). However, time of day affected the proportion of pecks that the blue and red circles received ($X^2_2 = 13.22$, $P = 0.0013$; $X^2_2 = 13.03$, $P = 0.0015$, respectively). The proportion of blue pecks were significantly increased at noon (Figure 5.2b) while the red proportion was significantly higher at dusk than midday (Figure 5.2a). Water color strongly affected fish

preference for blue. The number of trials recording blue pecks was significantly higher in tea-stained tanks ($X^2_1 = 44.51$, $P < 0.0001$). In fact, out of 36 blue pecks, all were in tea-stained tanks (Figure 5.2b). Water color also had a significant effect on green ($X^2_1 = 10.5$, $P = 0.001$) and orange ($X^2_1 = 4.15$, $P = 0.042$), where fish in clear tanks pecked significantly more at these colors than fish in tea-stained tanks (Figure 5.2c, 2d).

DISCUSSION

Opsin expression is dynamic

Our study shows that there are strong diurnal patterns of opsin expression in *Lucania goodei*. Opsin expression in both clear and tea-stained tanks rises substantially from morning to afternoon and then falls back to base levels after sunset. However, contrary to our expectations, opsin expression does not precisely match light levels, with highest expression at midday. Rather, the patterns of expression might better be described as a delayed bimodal reaction to changes in light levels, with up-regulation and down-regulation occurring only after prolonged exposure to rising or falling light levels. Of course, there is an inherent time delay between opsin expression and a change in cone phenotype. In rods, mRNA moves from the nucleus to the myoid region, where it forms opsin, within an hour (Bok 1970), and Bok and Young (Bok and Young 1972) found that it took from 30 minutes to two hours for radioactively labeled proteins to be incorporated into cone outer segments. That means the mRNA expression pattern that we observe here is probably related to outer segment growth approximately two hours subsequent. Nonetheless, our results suggest that opsin abundance, and therefore sensitivity, in the cones is highest in the late afternoon/early evening and lowest in the pre-dawn and early morning hours. This matches previous work on diurnal rhythms found in other fish; both zebrafish and the cichlid *Haplochromis burtoni* have cone opsin

expression that peaks in the late afternoon (Li and Dowling 1998; Halstenberg et al. 2005). In zebrafish, this peak in expression has been demonstrated to correlate with a peak in sensitivity to visual stimuli, with fish most sensitive to visual stimuli in the late afternoon and least sensitive in the early morning hours (Li and Dowling 1998; Li et al. 2005). This suggests that we have observed a general cross-taxa pattern where opsin expression does not march lock-step with light availability.

Throughout the day, the relative amounts of each wavelength of light also change in addition to changes in overall light levels, with light becoming blue-shifted at dawn and dusk. We sought to determine if proportional expression of the cone opsins matches these shifts. While proportional expression of SWS1 was highest in the morning, it was not matched by a corresponding proportional increase in the evening (Figure 5.1l), which would be indicative of increased utilization of the relatively abundant blue/UV wavelengths available at those times. The same pattern was found with the LWS opsin. Proportional LWS expression was highest at noon, when long-wavelengths are particularly abundant, but proportional expression remained high through the evening, after long-wavelength light had fallen. Therefore, our results do not indicate proportional opsin expression matches relative wavelength abundance.

Interestingly, we did find the temporal effects on expression were most pronounced in those opsins with λ_{max}'s that correspond to the most striking overall light shifts (i.e. the longer wavelengths). LWS and RH2-1, which maximally absorb yellow and green light, had very pronounced circadian rhythms, increasing from their low points (midnight and dawn) to high points (noon and dusk) an average of 2.6-fold and 2-fold, respectively when calculated relative to EF1-α (Figure 5.1a, 1b). These increases largely drove the patterns observed in the proportional data. On the other hand, RH2-2, SWS2A, SWS2B, and SWS1, which absorb in the blue to UV range, increased only 1.1-1.3 fold (Figure 5.1c-f), and the increase was not

significant in SWS1 or SWS2A (Table 5.1). Work on the cichlid *H. burtoni* also found smaller diurnal changes in expression in the short-wavelength sensitive opsin (SWS2A) than in middle (RH2) and long-wavelength (LWS) sensitive opsins. Considering that yellow light rises and falls much faster than blue light at dawn and dusk, it could be that cones sensitive to longer wavelengths are therefore much more affected by diurnal rhythms than shorter wavelength cones. On the other hand, the invariant production of SWS1 might also serve a purpose. Given that previous work has indicated that there are fewer SWS1 cones than LWS cones in *L. goodei* (Fuller et al. 2003), but SWS1 expression was actually higher than LWS expression in the morning, it seems likely that SWS1 cones maintain a consistently high (rather than consistently low) production of opsin throughout the day. There might be some advantage to maintaining a constant high level of opsin production in the shorter wavelengths if the ability to see predators, food, or mates during the transitionary times of dawn and dusk is especially important. For example, Munz and McFarland (Munz and McFarland 1973) have suggested that the rhodopsins of reef fish are attuned to evening twilight conditions because predation reaches a maximum during this transitionary time, although Endler's (Endler 1987) study on guppies, a freshwater species, indicated the opposite pattern of predation. We have very little data concerning temporal differences in predation, or mating or foraging behaviors, making it difficult to say whether dawn and dusk are key times of day for *L. goodei*. Fuller (Fuller 2001) examined male mating behaviors in the field and found a small effect of time on male-male aggression, with aggression peaking a couple hours after dawn, but the author attributed this peak to local boating activity suppressing behaviors in the afternoon.

Our results on opsin gene expression in tea-stained and clear water also point to the idea that SWS1 is less labile than the other opsins. All opsins, with the exception of SWS1, demonstrated increased expression in tea-stained water (Table 5.1, Figure 5.1) across all the

time points measured. This distinct behavior of SWS1 in tea-stained environments has also been indicated by previous research in the species. *L. goodei* populations have fewer SWS1 cones in tea-stained water (Fuller et al. 2003), which is caused in large part by a static expression of SWS1 opsin while the longer wavelength sensitive opsins up-regulate (Fuller and Claricoates 2011). Thus, short-wavelength opsins, especially SWS1, seem to be less influenced than other opsins by lighting environment as a whole, whether considering temporal or habitat factors. The reasons for this are unclear, but UV vision in fish is important for a variety of things such as predator and prey detection, navigation, identification of conspecifics, and avoidance of excessive UV exposure (Losey et al. 1999).

Behavioral color preference

We were interested in whether the opsin expression patterns that we observed were related to a measurable behavioral phenotype, in this case foraging preference for different colored circles. On a general level, expression of the LWS opsin was highest, and this corresponded to a high preference for red circles (and orange circles to a lesser extent) overall. *L. goodei* can be added to a long list of organisms that exhibit a preference for long-wavelength colors (Rodd et al. 2002; Smith et al. 2004; Ham and Osorio 2007). This bias could be the result of selection for increased consumption of carotenoids or related to sexual selection (males have red, yellow, and orange coloration, females do not).

The temporal opsin expression data indicates that the fish should be most attracted to red in the evening, and the fish did peck at red significantly more at dusk than at midday, while dawn was intermediate (Figure 5.2a). But based solely on expression data, the fish should also have been more attracted to blue in the morning, which we did not observe. Rather, we see a peak attraction to blue at midday (Figure 5.2b). These results suggest that attraction to colors in killifish is not based on opsin production, but rather on lighting conditions. The fish prefer red at dusk, and to a lesser extent dawn, times when less long-

wavelength light is available. At noon, when short wavelength light is at a relative minimum, the fish have a higher preference for blue. Irrespective of time of day, animals in tea-stained water, which transmits less short-wavelength light, also peck significantly more at the blue discs, perhaps at a cost of green and orange (Figure 5.2b-d).

The *L. goodei* preference for blue in tea-stained waters has been found elsewhere (Fuller et al. 2010), and when combined with the temporal data suggests that contrast is highly important to these fish. When there are low levels of short-wavelength light, as in either tea-stained waters or waters at midday, blue colors appear high in contrast and the fish demonstrate a high preference for blue. Color contrast such as this is highly important to many organisms (Leal and Fleishman 2002; Uy and Endler 2004; Thery et al. 2008; Gray et al. 2008; Dalton et al. 2010). Indeed, this strong preference for contrast seems to have affected the color patterns of the killifish. Males can have red, yellow, or blue anal fins, and there are more many more blue-morph males found in tea-stained water, where their contrast is higher (Fuller 2002). Our results also suggest a mechanism of maintenance of this polymorphism. Red and yellow males may have higher mating success at dawn and dusk, when they are most conspicuous to females, while blue males may have higher success at midday. This is an exciting avenue of research to continue. Nonetheless, the behavioral study shows that attraction to colors as measured by foraging preference does not match opsin expression, but rather correlates negatively with available light color.

This work supports previous results that found inter-individual differences in opsin expression were not correlated with pecking preference (Fuller et al. 2010). However, color preferences must be a function of opsin production and cone abundance at some level. Color perception relies on the differential stimulation of different cone classes. Presumably, deleting (or adding) an entire opsin class would alter color perception dramatically. Such wholesale shifts in opsin expression are seen over the course of development in many animals

(Evans and Fernald 1993; Cheng and Flamarique 2004; Cheng and Flamarique 2007; Shand et al. 2008; Taylor et al. 2011). Closely related species can use different templates of opsins (Carleton and Kocher 2001). Hence, it is not appropriate to say that opsin expression has no effect on color preferences. Still, our data here are consistent with the idea that short-term plastic shifts in opsin gene expression do not drive color preferences.

Conclusions

We have demonstrated that there are very large diurnal effects on cone opsin gene expression, but that the variation in expression does not precisely match lighting levels. It is striking how large these effects are, especially in comparison to known effects of habitat lighting environments. For example, LWS opsin increases 260% from midnight/dawn to noon/dusk. However, switching the fish's habitat to that of a tea-stained swamp elicits a much smaller increase (25%) in our study. This suggests that animals might be able to utilize standing temporal variation to easily alter their visual system to match the environment. Given the large effect of time on opsin expression, our results also illustrate the dangers of comparing opsin expression across populations or individuals without controlling for time of day. Our results indicate that lighting environment is far more important than diurnal opsin expression patterns in *Lucania goodei* in regard to behavioral impact. Color preference is highly influenced by natural variation in lighting conditions. When short wavelengths are abundant (dawn and dusk) red is preferred, when they are proportionally scarce (noon) blue is a preferred color. In these fish, color contrast is highly important.

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Table 5.1. Analysis of variance results on opsin values relative to EF1-α and on proportional opsin values. Day and the qRT-PCR plate each individual was run on are included as random effects. Values relative to EF1-α were log transformed. λ_{max} values are from [22,25,30].

	relative to EF1-α (opsin/EF1-α)				**proportional (opsin/all opsins)**			
	time		**water**		**time**		**water**	
	$F_{3,57}$	**P**	**$F_{1,57}$**	**P**	**$F_{3,57}$**	**P**	**$F_{1,57}$**	**P**
LWS (λ_{max} = 573)	31.72	**<0.0001**	5.09	**0.028**	33.16	**<0.0001**	4.05	**0.049**
RH2-1 (λ_{max} = 539)	21.61	**<0.0001**	13.63	**0.0005**	1.89	0.14	15.73	**0.0002**
RH2-2 (λ_{max} =455)	3.21	**0.030**	7.98	**0.0065**	13.06	**<0.0001**	4.41	**0.040**
SWS2A (λ_{max} ~448)	1.78	0.16	4.42	**0.040**	11.53	**<0.0001**	3.7	0.059
SWS2B (λ_{max} = 405)	8.43	**<0.0001**	8.45	**0.0052**	11.97	**<0.0001**	0.93	0.34
SWS1 (λ_{max} = 359)	2.06	0.12	2.08	0.15	21.46	**<0.0001**	23.03	**<0.0001**

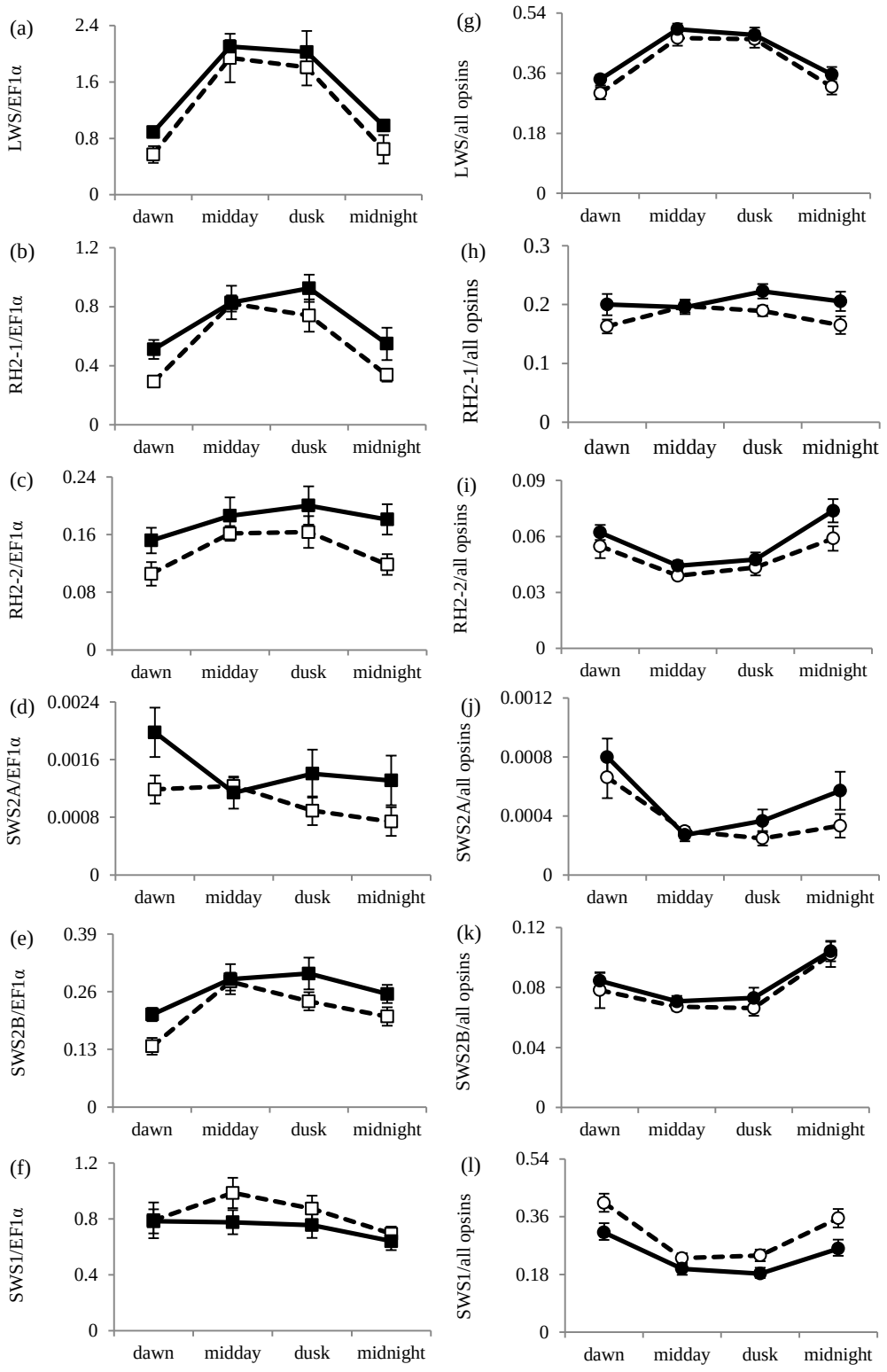

Figure 5.1. Opsin expression relative to housekeeping gene EF1-α (a-f) and proportional to all opsins (g-l). Open symbols are means (± standard error) of individuals from clear tanks. Closed symbols are means (± standard error) of individuals from tea-stained tanks.

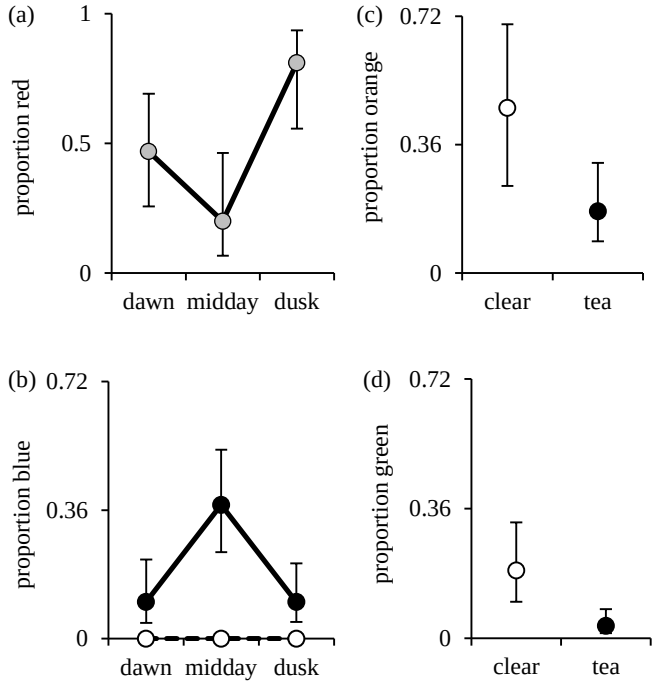

Figure 5.2. Proportion of pecks that were red (a), blue (b), orange (c), or green (d) as a function of either time of day or water color. Six tanks of 14 fish were given the opportunity to peck at red, orange, yellow, green, blue, black or white spots. Black symbols are for tea-stained water, open symbols are for clear water, and grey symbols are from combined data. Error bars represent 95% confidence intervals.

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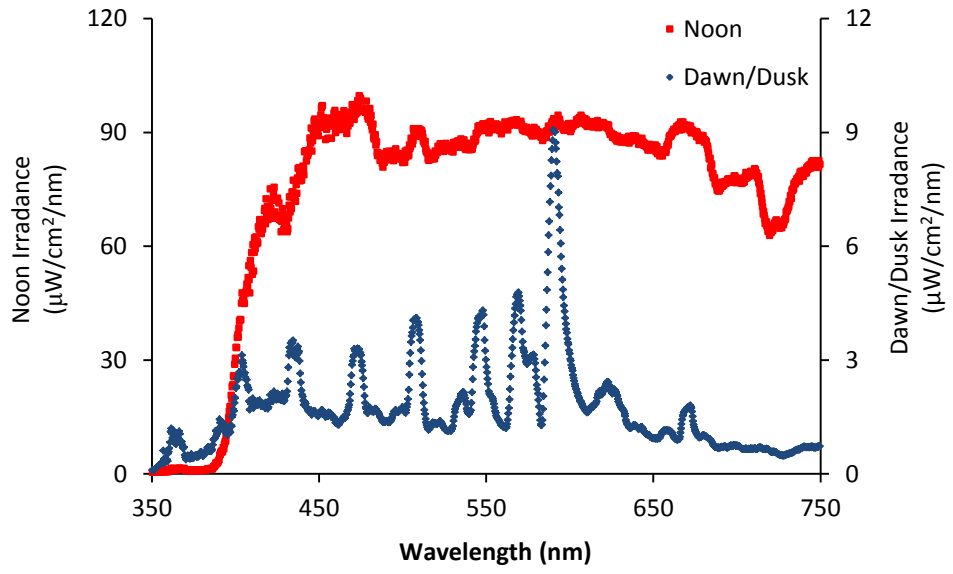
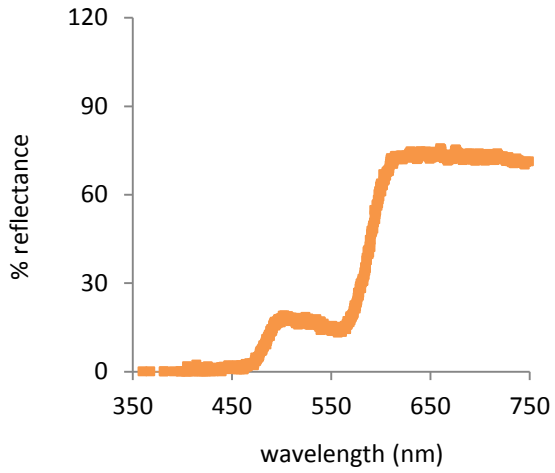
Supplemental Table 5.1- χ^2 and P values for the effects of time (dawn, midday, and dusk) and water color (tea and clear) on proportion of each color the fish pecked at. Black and white are not included as they only received 1 and 3 pecks total respectively.

	time		water	
	χ^2	P	χ^2	P
red	11.9	**0.0026**	1.52	0.22
orange	5.32	0.07	4.21	**0.04**
yellow	3.46	0.18	0.37	0.54
green	3.58	0.17	9.27	**0.0023**
blue	13.75	**0.001**	21.01	**<.0001**



Supplemental Figure 5.1- Typical irradiance spectra at noon and dawn/dusk. Readings were taken at surface level of the tanks using an Ocean Optics USB2000 spectrophotometer. Note the order of magnitude difference in scale.



Supplemental Figure 5.2- Reflectance of orange dots.